

THE ROLE OF HSV-1 PROTEIN ICP22 IN TRANSCRIPTION

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Herpes Simplex Virus type 1 (HSV-1) productively replicates in epithelial cells before establishing a life-long infection in sensory neurons. During productive infection, HSV-1 alters cellular processes and hijacks many cellular proteins to aid in viral replication. Viral gene transcription requires cellular RNA polymerase II (RNA pol II) and is highly regulated. The viral Immediate Early (IE) proteins ICP4, ICP0, ICP27, and ICP22 have all been recognized to play roles in regulating viral transcription. To clarify the role of ICP22 in transcription regulation, we defined its interactions with cellular transcription factors and its effects on both viral and cellular gene transcription. We determined that: i) the FACT complex, a cellular transcription elongation factor, did not associate with viral DNA in the absence of ICP22; ii) in the absence of ICP22, viral transcription decreases and RNA pol II does not efficiently proceed from viral promoters to gene bodies; iii) ICP22 directly interacts with the FACT complex and other cellular factors involved in transcription; and iv) ICP22 is required for the efficient repression of cellular gene transcription.

Our studies suggest a role for ICP22 in regulating transcription in cells infected with HSV-1. We propose that ICP22 recruits cellular factors necessary for efficient transcript elongation to viral genomes. Furthermore, the ability of ICP22 to recruit these factors supports a mechanism through which HSV-1 limits cellular transcription while encouraging robust viral transcription. ICP22 is therefore integral to the efficient production of infectious virus and the sustained evasion of host immune responses.

TABLE OF CONTENTS

1.0	INTRODUCTION.....	1
1.1	PATHOLOGY AND HUMAN HEALTH.....	1
1.1.1	Herpesviridae.....	1
1.1.2	Pathology.....	2
1.1.3	HSV Vectors	3
1.2	GENERAL BIOLOGY AND HSV-1 LIFECYCLE.....	4
1.2.1	Virion Structure	4
1.2.2	Genome Structure	5
1.2.3	Productive Infection: Overview	6
1.2.4	Productive Infection: Entry into Cells	7
1.2.5	Productive Infection: Detection by the Cell.....	7
1.2.6	Productive Infection: Transcription.....	10
1.2.7	Productive Infection: DNA Replication and Recombination.....	12
1.2.8	Productive Infection: Packaging and Egress.....	13
1.2.9	Latency	14
1.3	CELLULAR RESPONSES TO HSV-1 INFECTION.....	16
1.3.1	Innate Immunity	16
1.3.2	Adaptive Immunity	17

1.3.3	Viral Evasion of Immune Responses	17
1.4	HSV-1 AND TRANSCRIPTION	20
1.4.1	Viral Proteins Involved in Transcription.....	20
1.4.2	Cellular Transcription Factors Associated with Viral DNA.....	23
1.5	RATIONALE.....	26
2.0	A HERPESVIRAL IMMEDIATE EARLY PROTEIN PROMOTES TRANSCRIPTION ELONGATION OF VIRAL TRANSCRIPTS.....	27
2.1	PROJECT SUMMARY.....	27
2.2	IMPORTANCE	28
2.3	INTRODUCTION	29
2.4	RESULTS.....	31
2.5	SUPPLEMENTAL FIGURES	46
2.6	DISCUSSION.....	48
2.7	MATERIAL AND METHODS.....	51
3.0	THE ROLE OF ICP22 IN THE INHIBITION OF CELLULAR TRANSCRITPION DURING HSV-1 INFECTION	58
3.1	PROJECT SUMMARY.....	58
3.2	IMPORTANCE	59
3.3	INTRODUCTION	59
3.4	RESULTS.....	62
3.5	DISCUSSION.....	78
3.6	MATERIALS AND METHODS.....	82
4.0	SUMMARY AND DISCUSSION	86

4.1 SUMMARY OF THESIS.....	86
4.2 GENERAL DISCUSSION.....	88
BIBLIOGRAPHY.....	92

LIST OF TABLES

Table 1. Proteins Associated with Viral Genomes.....	37
Table 2. Proteins interacting with ICP22 at 6 hpi	78

LIST OF FIGURES

Figure 1. The HSV-1 Virion.	4
Figure 2. HSV-1 Genome Structure.....	5
Figure 3. HSV-1 Productive Lifecycle.	6
Figure 4. The roles of ICP4, ICP27, and ICP22 in HSV-1 transcription.....	22
Figure 5. Abundance of FACT complex proteins and localization of SSRP1 in infected cells..	34
Figure 6. Abundance of FACT complex proteins and localization of SSRP1 in infected cells. .	35
Figure 7. Interaction of ICP22 and the FACT complex in infected cells.	39
Figure 8. Replication and gene expression of an ICP22 mutant.....	41
Figure 9. ChIP analysis of ICP4 and RNA Pol II on ICP22 mutant versus wt (KOS) genomes.	43
Figure 10. Changes to gene expression in cells infected with HSV-1 with or without ICP22 over the course of productive infection.....	63
Figure 11. Differences in pathway regulation due to HSV-1 infection at 2 hours post infection.	66
Figure 12. Differences in pathway regulation due to HSV-1 infection at 6 hours post infection.	69
Figure 13. RNA polymerase II occupancy on cellular and viral genes during HSV-1 infection.	72
Figure 14. Kinase inhibitor DRB alters phosphorylation of RNA pol II.	74
Figure S1. Total ChIPseq Reads.....	46
Figure S2. Calculation of Start Site and Gene Body Reads for Selected Genes.....	47

LIST OF ABBREVIATIONS

Asf1: Anti-silencing function 1 histone chaperone

bp: Base Pair

Cdk (7/9): Cyclin dependent kinase (7/9)

ChIP-seq: Chromatin Immunoprecipitation Sequencing

CK2: Caesin Kinase 2

coREST: REST Co-repressor protein

CTD: C-terminal Domain

DNA-PK: DNA damage response kinase

dsDNA: Double-stranded DNA

DSIF: DRB-sensitivity-inducing factor

E: Early

EBV: Epstein-Barr virus

EdC: 5-Ethynyl-2'-deoxycytidine

EIF: eukaryotic initiation factor

FACT Complex: Facilitates Chromatin Transcription Complex

g(B-L): Glycoprotein (B-L)

GFP: Green Fluorescent Protein

HAT: Histone acetyl transferase

HCF-1: Host Cell Factor-1

HCMV: Human Cytomegalovirus

HDAC: Histone deacetylase

HHV-6A: Human Herpesvirus 6A

HHV-6B: Human Herpesvirus 6B

HHV-7: Human Herpesvirus 7

HSV-1: Herpes Simplex Virus 1
 HSV-1: Herpes Simplex Virus Type 1
 HSV-2: Herpes Simplex Virus 2
 HVEM: Herpes Virus Entry Mediator
 ICP: Infected cell polypeptide
 IE: Immediate Early
 IFI: Interferon-inducible
 IFN: Interferon
 IL: Interleukin
 INR: Initiator element
 iPOND: isolation of proteins on nascent DNA
 IRF: Interferon Regulatory Factor
 ISG: Interferon-stimulated Gene
 JMJD3: Lysine-specific demethylase 6B
 KSHV: Kaposi's Sarcoma-associated Herpesvirus
 L: Late
 LAT: Latency Associated Transcript
 LSD1: Lysine-specific histone demethylase 1A
 MHC: Major histocompatibility complex
 miRNA: Micro RNA
 MOI: multiplicity of infection
 mRNA: messenger RNA
 ND10: Nuclear Domain 10
 NELF: Negative Elongation Factor
 NF1: Nuclear Factor 1
 NF κ B: nuclear factor kappa-light chain-enhancer of activated B cells
 NURD: Nucleosome Remodeling and Deacetylase
 Oct1: Octamer binding protein 1
 oriL: Origin L
 oriS: Origin S

PML: Pro Myelocytic Leukemia
PRR: Pattern Recognition Receptor
RNA-seq: RNA sequencing
RPA: replication factor-A protein
Ser-2P: Serine-2 Phosphorylated
Ser-5P: Serine-5 Phosphorylated
Set1/MLL: Histone-lysine N-methyltransferase
Sin3A: Paired amphipathic helix protein
Sp1: Specificity Protein 1
Spt: Suppressor of Ty
ssDNA: Single-stranded DNA
SSRP1: Structure Specific Recognition Protein 1
STING: Stimulator of interferon Genes
SWI/SNF: Switch/Sucrose Non Fermentable
TAF: TBP Associated Factor
TBK: TANK-binding kinase
TBP: TATA-binding protein
TF: Transcription Factor
tk: thymidine kinases
TLR: Toll-like Receptor
U_L: Unique long region
U_S: Unique short region
UTX: Lysine-specific demethylase 6A
VHS: virion host shutoff protein
VP: Viral Protein
VZV: Varicella-zoster Virus
WT: Wild Type

1.0 INTRODUCTION

Herpes Simplex Virus (HSV) is a ubiquitous human pathogen that can produce mild to severe pathologies. HSV is also employed as a therapeutic vector. A deeper understanding of the basic effects of HSV proteins and processes on the cell is essential for developing safe translational applications. Here we elucidate the role of viral protein ICP22 in viral and cellular transcription during productive viral infection. The interactions of ICP22 with cellular proteins throughout infection also illuminate the dynamics of protein interactions in infected cells.

1.1 PATHOLOGY AND HUMAN HEALTH

1.1.1 Herpesviridae

The family of Herpesviridae consists of a multitude of viruses that infect a wide range of animals. Herpesviruses are categorized as such based on their morphology: a double-stranded DNA genome encased in an icosahedral capsid. Nine herpes viruses infect humans: Herpes Simplex Virus 1 (HSV-1), Herpes Simplex Virus 2 (HSV-2), Varicella-zoster Virus (VZV), Human Cytomegalovirus (HCMV), Epstein-Barr virus (EBV), Human Herpesvirus 6A (HHV-6A), Human Herpesvirus 6B (HHV-6B), Human Herpesvirus 7 (HHV-7), and Kaposi's Sarcoma-associated Herpesvirus (KSHV) (1). Although not considered a human pathogen,

zoonotic infection of Cercopithecine Herpesvirus 1 (B virus), a common virus in Asian macaques, has occurred and usually results in fatal encephalomyelitis (2). Herpesviridae can be divided into three subsets: Alphaherpesviruses, Betaherpesviruses, and Gammaherpesviruses. HSV-1, HSV-2, and VZV are considered to be Alphaherpesviruses, sharing common characteristics such as a variable host range, short reproductive cycle during productive infection, and the establishment of latency in sensory ganglia. HSV-1 and HSV-2 both initially infect cells at mucosal surfaces or sites of broken skin (1). About 17% of the genomes of HSV-1 and HSV-2 are different and infections can be serologically differentiated (3). HSV-1 is often identified as the cause of oropharyngeal infection, while HSV-2 is most often identified in genital infections. A primary infection of either HSV-1 or HSV-2 at one site does not preclude primary infection by the other at a different site (1). This project will focus on HSV-1.

1.1.2 Pathology

HSV establishes a lifelong infection in its host. According to recent surveys conducted in the United States, 47.8% of people aged 14-49 are infected with HSV-1 and 11.9% with HSV-2. The prevalence of both increased with age and was higher among women (4). HSV-1 infection gives rise to a range of clinical symptoms. Although many patients experience only recurrent cold sores, HSV-1 infection can cause morbidity and mortality(5). Herpes Stromal Keratitis, which causes blindness, affects 300,000 new patients yearly in the United States(6). Neonatal herpes and HSV encephalitis can both have fatal outcomes (5). The prevalence of HSV-1 in pregnant women is 59.3% HSV-1 and 21.1% are seropositive for HSV-2, and the risk of neonatal herpes increases if primary infection occurs near delivery (7). Antiviral therapies have been developed that target the viral DNA polymerase or viral thymidine kinase (tk). Acyclovir, a nucleoside

analogue, is an effective antiviral that is used to treat both mild and severe HSV-1 infections (8). Unfortunately, HSV-1 can develop resistance to acyclovir treatment, especially in immunocompromised patients. HSV-1 infections in immunocompromised patients are caused by acyclovir resistant strains in 3.5%-10% of cases depending on the nature of the infection. While second-line drugs are available, they are only available by intravenous administration and have severe side effects (9).

1.1.3 HSV Vectors

Although HSV is generally recognized as pathogenic in humans, modified HSV has been widely explored as an important tool of gene therapy. There are multiple benefits of using HSV, including: the ability to infect neurons; rapid replication in cell culture; ease of genetic modification; lack of integration into host genomes; and the size of genes that can be delivered. Various types of vectors are used for different applications. Attenuated mutants allow for replication in specific cells, such as rapidly replicating cancer cells. Genes deleted in these cases include thymidine kinase (tk) and γ 34.5. Replication defective vectors can fail to replicate due to the deletion of genes such as those which code for ICP4, but still remain cytotoxic due to the expression of the remaining viral genes. Further deletion of Immediate Early Viral Proteins can eliminate cytotoxicity. Amplicon vectors package an amplicon, or plasmid, within a virion by providing the HSV origin of replication and other HSV DNA packaging signals (10).

The most attractive application of HSV vectors is in the treatment of brain tumors such as glioblastoma (11). There have been many clinical studies using oncolytic HSV vectors to treat Malignant Glioma. HSV vectors used in these studies most often have a deletion of γ 34.5 and exhibit no toxicity in human subjects (12). Recently work has been done to retarget HSV vectors,

restricting the vector's ability to infect cells in order to target a specific type (13). Retargeting will allow HSV vectors to be useful in treating even more conditions.

1.2 GENERAL BIOLOGY AND HSV-1 LIFECYCLE

1.2.1 Virion Structure

The HSV-1 virion, illustrated in Figure 1, is composed of a core containing viral dsDNA. HSV-1 is a large virus with a dsDNA genome encapsulated by an inner core, an icosahedral capsid, a proteinaceous tegument, and a lipid envelope complete with glycoprotein spikes (14).

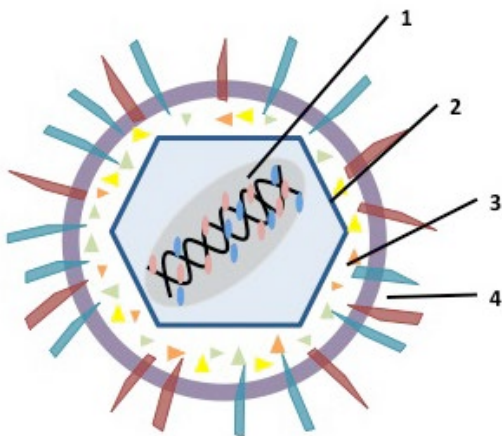


Figure 1. The HSV-1 Virion. 1. Core containing viral DNA bound by spermidine and spermine. 2. Icosahedral capsid. 3. Tegument containing tegument proteins. 4. Viral envelope studded with glycoproteins.

In addition to viral DNA the core contains spermidine and spermine tightly bound to the DNA, which neutralize the DNA phosphate (15). The viral capsid is composed of an outer layer with a T=16 icosahedral symmetry and is composed of the viral structural proteins VP5, VP26,

VP23, and VP19C (16, 17). The tegument of the virus, between the capsid and the envelope, contains at least 18 viral proteins that are released into infected cells upon entry. The envelope also contains a multitude of viral proteins embedded in the membrane (14). Cryo-Electron Tomography studies have revealed specifics about the tegument and envelope and an estimation of the size of HSV-1 virions. Diameters measured ranged from 170 to 200 nm with a bilayer membrane envelope about 5 nm thick. Including the glycoprotein spikes, of which there 600 to 750 per virion, the total diameter is about 225 nm (18).

1.2.2 Genome Structure

The HSV genome encodes around 80 genes and is approximately 152 kb (19). The genome is GC rich and is composed of a unique long region (U_L), a unique short region (U_S), long repeat regions, and short repeat region (20–23) (Figure 2). The repeat regions flank their respective unique regions and facilitate recombination during viral DNA replication. The regions invert, resulting in four possible orientations of the genome. All four isomers are generated during normal replication (24). The genome contains three origins of replication, one copy of $oriL$, within the U_L region, and two copies of $oriS$, within each one of the short repeat regions (25, 26). Mutation of the $oriL$ or $oriS$ does not prevent replication, indicating that possessing all three origins is not required for replication in culture nor for pathogenesis in a mouse model (27, 28).



Figure 2. HSV-1 Genome Structure. A schematic including the locations of origins of replication.

1.2.3 Productive Infection: Overview

HSV-1 enters the body through the mucosa and primary productive infection ensues in epithelial cells. After entry, the virus undergoes transcription, replication, and packaging within the nucleus (14). Productive infection is rapid, with some virus beginning to undergo packaging and export by 6 hours post infection (29). An outline of these steps is shown in Figure 3.

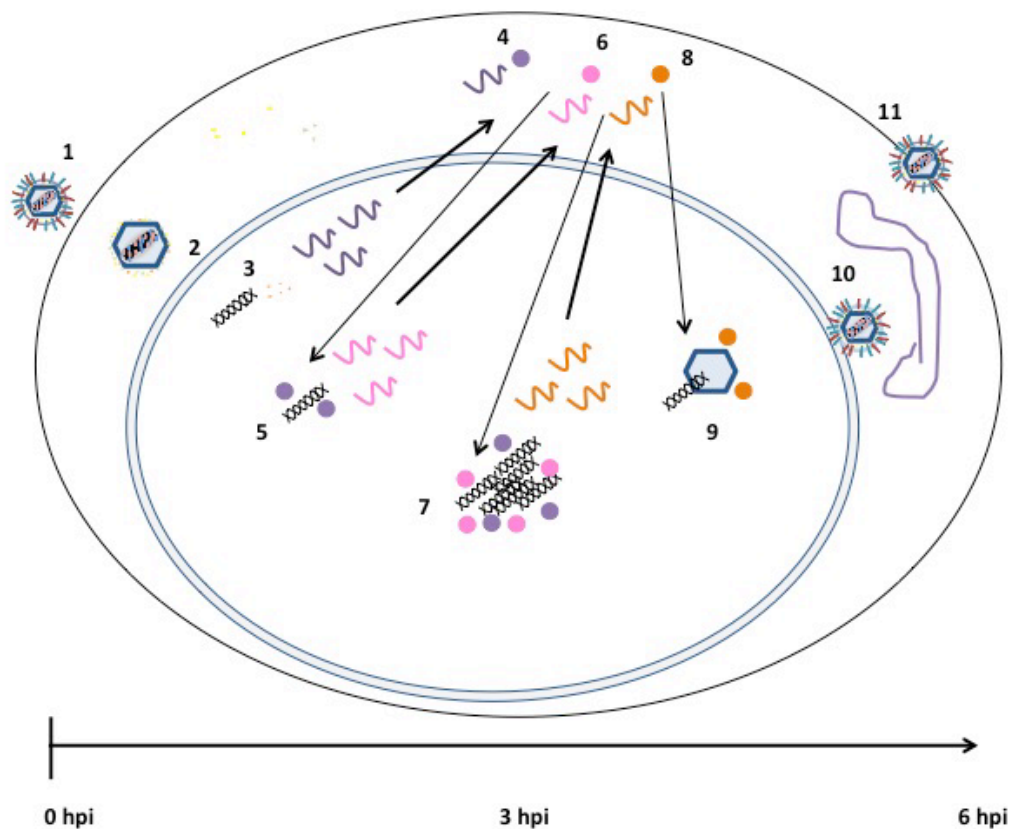


Figure 3. HSV-1 Productive Lifecycle. Timeline indicates approximate initiation of each event. 1. Entry into cell through fusion with cell envelope or endosome. 2. Translocation of capsid and tegument proteins to nucleus. 3. Transcription of IE genes. 4. Translation of IE proteins. 5. Transcription of E genes. 6. Translation of E proteins. 7. Viral DNA replication and transcription of L genes. 8. Translation of L proteins. 9. Packaging of DNA into capsids. 10. Egress from nucleus. 11. Acquisition of envelope and egress from cell.

1.2.4 Productive Infection: Entry into Cells

HSV-1 enters epithelial cells through a complex interaction between multiple viral glycoproteins and cellular receptors. Depending on cell type, the virion will enter the cell through membrane fusion between its envelope and the cell surface or through endocytosis. In either case, the viral glycoproteins required—gB, gD, and gH-gL—are the same (30, 31).

HSV-1 will first interact with the cell surface through glycoprotein gC or gB binding to cellular heparin sulfate. Next, gD, which is essential for entry, will bind independently to a cellular receptor. Three independent cellular receptors have been identified: Nectin-1, HVEM, and 3-O-HS (modified heparin sulfate). After gD binds to a receptor, gB and gH-gL can initiate fusion (32). The capsid, along with associated tegument proteins, can then traffic to nuclear pores and release viral DNA into the nucleus (33). The tegument proteins can have an immediate effect on the cell and many are required to continue the viral lifecycle.

1.2.5 Productive Infection: Detection by the Cell

As HSV-1 enters host cells, it usurps and perturbs normal cellular processes. The cell responds to these disturbances in multiple ways. Here we will discuss the three ways the cell interacts with the incoming virus: the DNA damage response, intrinsic immunity, and histone deposition.

The HSV-1 genome that enters the cell nucleus has nicks and gaps (34, 35). The genome is recognized in the host by the DNA damage response, with proteins such as PARP1, PARP14, RPA1, and LIG3 associating with viral DNA as soon as one hour post infection (29). Virion DNA transfected into cells stimulates DNA damage response kinases (DNA-PKcs), and more stimulation reduces the infectivity of HSV-1. While the DNA damage response could inhibit

HSV-1 infection, the viral Immediate Early (IE) protein ICP0 can degrade DNA-PKs, allowing the virus to evade this cellular defense (36).

The intrinsic immune response to HSV-1 involves the association with ND10 domains and the deposition of PML onto viral DNA upon entry into the nucleus (37, 38). Within PML nuclear bodies, repressive proteins, including Sp100, associate with viral genomes (29). Viral Immediate Early (IE) protein ICP0 disrupts ND10 domains by degrading PML and other repressive proteins, negating their repressive action on the viral genome (39–41).

Histones regulate access to DNA and play a role in transcription and replication of the DNA. Histones strongly associated with to DNA can prevent access by transcriptional or replicative machinery. Upon entry into the nucleus, HSV-1 DNA associates with histones as early as one hour post infection (29, 42, 43). On cellular DNA, histones form chromatin. Chromatin is a macromolecular complex made up of nucleosomes, which are normally regularly spaced on cellular DNA. Each nucleosome is comprised of 145-147 bp of DNA wrapped around histone octamers comprised of two each of the histone proteins H2A, H2B, H3, and H4 (44). Histones on viral DNA do not form chromatin identical to that on cellular DNA, but rather form disordered nucleosomes and have modifications related to active transcription (45–47). Viral and cellular proteins have been shown to modify histones on, or remove histones from, viral DNA, promoting viral gene expression and replication during productive infection. Chromatin modification can result in histones that are more or less associated with DNA resulting in the transcription of associated genes being either activated or repressed.

The tegument proteins VP16 and VP22 interact with chromatin modifiers upon entry. HCF-1, with which VP16 forms a complex, recruits many chromatin modification complexes including Asf1, LSD1, Set1/MLL (48, 49). Asf1 may deposit H3.3 on viral genomes, LSD1 can

remove the repressive methylation from H3K9, and Set1/MLL can methylate H3K4 (a mark of activation), all of which lead to a more open chromatin structure on the genome. Transfected VP16 acidic activation domain (VP16 AD) has been shown to recruit histone acetyl transferase (HAT), which promotes activating acetylation of histones, and SWI/SNF, an ATP-dependent nucleosome remodeler which removes histones from DNA, to condensed chromatin in cell culture (50). Furthermore, the VP16 AD has been found to promote the association of HATs p300 and CBP, as well as ATP-dependent remodeling proteins BRG1 and hBRM, with viral IE gene promoters during lytic infection. Additionally, fewer H3 histones associate with viral IE gene promoters in the presence of the VP16 AD (51). VP22, another viral tegument protein, binds to template-activating factor I, a chromatin remodeling protein, *in vitro*, suggesting a possible role for VP22 in preventing template-activating factor I from depositing histones onto viral DNA (52).

It has been observed that in the presence of IE gene products, fewer histones associate with E and L gene promoters (42). This is consistent with a model of latency in which a mutant lacking all the IE genes (d109) can establish a quiescent infection in stationary cells in which viral DNA is silenced by heterochromatin (53, 54). ICP0, an IE protein, has an important role in modifying chromatin on viral genomes. Expression of ICP0 during infection has been found to increase levels of acetylated H3 on viral genomes at 6 hours post-infection (hpi) (43). ICP0 also has a role in removing histone H3 from IE and E genes between 3 and 8 hpi, potentially by recruiting histone remodeling complexes and/or promoting histone acetylation (43). One way ICP0 may promote activating histone marks is by dissociating histone deacetylases from the CoREST-REST complex, allowing LSD1 to demethylate the repressive methylation of H3K9 rather than removing the activating methylation of H3K4, and by inhibiting class II histone

deacetylases, maintaining activating acetylation of histones (55, 56). ICP4, another IE protein and a transcription activator, while not directly implicated in chromatin modification, has been found to interact with the chromosomal protein high mobility group protein 1 and the ATPase chromatin remodeling complexes INO80, NURD, and SWI/SNF (57, 58).

1.2.6 Productive Infection: Transcription

In the nucleus, HSV-1 genes are transcribed by RNA polymerase II (RNA pol II) in a regulated cascade (59). Gene classification is determined based on when the genes are expressed, and they naturally cluster by function. Immediate early (IE) genes are expressed in the absence of de novo viral protein synthesis. IE gene products mainly regulate transcription (60). Next expressed are early (E) genes, whose products are involved in viral DNA replication. The initial rounds of viral DNA replication allow for the expression of late (L) genes, and this expression peaks after the accumulation of viral genomes. L genes encode mainly structural proteins and tegument proteins. L genes that may be expressed at low levels prior to viral DNA replication are considered to be leaky late genes (γ_1) and those that are strictly expressed after the onset of DNA replication are considered to be true late genes (γ_2) (61).

IE genes are transcribed shortly after infection and expressed by one hour post infection, with peak transcription between 2 and 4 hpi (60, 62). Out of the five immediate early proteins, ICP4, ICP22, ICP27, and ICP0 associate with viral genomes by 2 hours post infection (29). The viral tegument protein VP16 forms a complex with the cellular protein Oct1 and the cellular coactivator Host Cell Factor-1 (HCF-1) in order to activate transcription of IE genes. The VP16 complex binds IE gene promoters via a TAATGARAT sequence (63). The gene promoters also have binding sites for the cellular transcription factors Sp1, NF1, and TFIID (64,

65). VP16 is required for the recruitment of RNA pol II, chromatin remodelers, and transcription factors to IE gene promoters (section 1.2.5) (66–72). The IE protein ICP4 plays a role in repressing the transcription of ICP4 and the other IE proteins at later times post infection (73, 74).

E genes are highly transcribed between 4 and 6 hours post infection, and their transcription requires the expression of the IE protein ICP4. The promoters of E genes do not have a VP16 binding site, but do contain a TATA box and binding sites for Sp1 and NF1 (75). Despite the Sp1 binding site, ICP4 may serve to provide the same function as Sp1 does. Mutation of the Sp1 binding site does not greatly affect E gene expression kinetics (76).

L genes are expressed after the onset of viral DNA replication, 3 hours post infection, and highly expressed around 6 hours post infection. The promoters of both leaky late and true late genes contain INR elements and TATA boxes (62). The IE proteins ICP27 and ICP22 aid in the transcription of L genes although the exact mechanism is not fully defined (77–79).

The transcription of E and L genes, which requires activation by ICP4, involves other IE genes and many cellular co-factors. ICP4 and these other factors are discussed in more detail in section 1.4.

HSV-1 proteins are translated by cellular translation machinery. HSV-1 increases the efficiency of viral protein translation by phosphorylating EIF4E and also promoting the assembly of EIF4F complexes by phosphorylating the EIF4E binding protein and leading to its degradation (80).

1.2.7 Productive Infection: DNA Replication and Recombination

HSV-1 encodes seven proteins required for viral DNA replication: UL9, ICP8, UL5, UL8, UL52, UL30, and UL42 (81). UL9, the origin binding protein, binds to the A-T rich origins of replication via specific binding sites. Two binding sites flank OriS while OriL is flanked by four, two on each side of the origin. These binding sites are palindromic, allowing for the formation of potential hairpins as the DNA is distorted in preparation for replication (82). There is also evidence that UL9 non-specifically binds ssDNA (83). While UL9 is required for origin dependent DNA replication early in infection, it is not required for continued DNA synthesis (84). Despite being dispensable for continued DNA synthesis, UL9 remains associated with viral DNA after the onset of DNA replication (29, 85). ICP8, the major viral single-strand binding protein binds nonspecifically to ssDNA. UL9 and ICP8 together distort the origins allowing for the start of replication and recruiting the helicase primase complex composed of UL5, UL8, and UL52 (86, 87). HSV-1 DNA synthesis is completed by the viral DNA polymerase, composed of UL30 and the processivity factor UL42 (88, 89).

Although the seven viral proteins described above are sufficient to promote origin dependent replication in vitro and in cell culture, in the context of productive infection many other viral and cellular proteins are involved in DNA replication and related processes such as recombination and nucleotide pool growth. Viral proteins involved in viral DNA replication but not required include thymidine kinase (TK), a ribonuclease reductase (ICP6, RR1, RR2), a uracil-DNA glycosylase (UNG), a deoxyuridine triphosphatase (dUTPase), and an alkaline nuclease (82). Cellular proteins involved in single strand break repair, double strand break repair, and mismatch repair associate with viral replication forks, indicating that DNA damage repair and/or recombination take place concurrent with viral DNA replication (85).

There are currently two proposed models for HSV-1 replication. The first, a rolling circle model, is based on the production of concatemeric DNA during replication (14). Recent evidence supports a model of replication linked to recombination (85, 90).

1.2.8 Productive Infection: Packaging and Egress

HSV-1 DNA is packaged into capsids in the nucleus. The capsid is composed of the structural proteins VP5, VP19C, VP23, and VP26 (91). The capsid also contains minor capsid proteins UL6, UL15, UL17, UL25, UL28, and UL33. The capsid develops into three forms in infected cells: (A) empty, (B) containing scaffold protein VP22a, (C) containing viral DNA (92). B-capsids will become either A-capsids or C-capsids, and C-capsids will mature into infectious virus (93). The icosahedral capsid is mainly composed of capsomers, which have either five or six copies of VP5 (91). During assembly, the scaffold protein VP22a interacts with VP5 and supports capsid assembly. In addition to the major capsid proteins, a portal complex, composed of 12 copies of the portal protein UL6, is incorporated into the capsid (94, 95). After assembly, VP22a is digested by the viral protease VP24 (96).

DNA packaging of HSV-1 requires at least seven viral genes: UL6, UL15, UL17, UL25, UL28, UL32, and UL33. The terminase complex, comprised of UL28, UL15, and UL33, docks on the capsid portal and binds to concatemeric viral DNA through the recognition of specific packaging sequences by UL28. Cleavage at these sites, *pac1* and *pac2*, ensure a single genome is packaged by the complex into each capsid. UL15 and UL17 are necessary to retain HSV-1 DNA into the capsid once it has been packaged (97).

Once DNA is packaged in capsids, the capsids bud from the nucleus, acquiring an envelope studded with viral glycoproteins from the inner nuclear membrane. These envelopes

then fuse with the outer nuclear membrane, releasing the capsid into the cytoplasm where it acquires tegument proteins. The capsids then bud into the Golgi, trans Golgi network, or endosome to acquire their permanent envelope, again studded with viral glycoproteins. Enveloped HSV-1 virions are transported in cytoplasmic vesicles to the cell surface, where the vesicles fuse with the plasma membrane and release the mature virion (98).

1.2.9 Latency

HSV-1 establishes a lifelong latent infection in sensory neurons from which it can periodically reactivate (99). After primary productive infection in epithelial cells HSV-1 can gain access to neuronal cells. Virions travel up axons to neuronal nuclei where the genome is maintained in a (mostly) quiescent episomal state (100, 101). The establishment and maintenance of latency in neuronal nuclei is attributed to various factors. First, although tegument proteins traffic to the nucleus of epithelial cells with the viral capsid, the distance between the cellular membrane and the nucleus is greater in neurons, and there is evidence that the tegument protein VP16 is often lost before the capsid reaches the nucleus (102). Additionally, HCF-1, which in epithelial cells forms a complex with VP16 to help initiate viral transcription, is located in the cytoplasm of neurons, separating it from incoming viral DNA (103). These two circumstances contribute to a lack of IE gene expression, preventing the initiation of the infectious cycle. Additionally, a host microRNA present in neurons—miR-138—repressed ICP0 expression (104). Two factors that contribute to the maintenance of latency are heterochromatin and the expression of latency associated transcripts (LATs). Latent genomes are associated with heterochromatin, characterized by the repressive histone modifications detected over most of the viral genome, repressing viral gene expression (105–107). One location of sustained transcription on latent

viral genomes is the region encoding LATs (108, 109). LATs are transcribed first as a 8.5 kb polyadenylated transcript, then spliced to produce a stable 2.0 kb intron, and some cases spliced further to produce other LATs. Among other roles, LATs can repress gene expression of genes such as the one which encodes ICP0. Expression of ICP0 is known to promote reactivation (110, 111).

Reactivation from latency is clinically complex, occurring in human patients due to various stressors such as fever and UV exposure (112, 113). Histone modification has been shown to induce reactivation of latent viral genomes. For example, inhibiting LSD1, JMJD3, or UTX, all of which modify chromatin to be less repressive, results in lower levels of HSV-1 reactivation (114, 115). On the other hand, HDAC inhibitors induce reactivation. HDAC inhibitors can also lead to an increase in ICP0, which may be vital to reactivation (116–118).

Reactivation involves a delicate balance, as the virus must stimulate the production of infectious virus while evading host responses to viral activity. The process of reactivation is therefore complex and encompassess multiple stages. Reactivation can be modeled by the addition of chemical compounds. After the induction of reactivation by chemical means such as treatment with phosphatidylinositol 3 (PI3)-kinase inhibitor, reactivation can be observed to take place in two phases. Phase I is characterized by the simultaneous transcription of IE, E, and L genes and occurs 15-20 hours post treatment. This transcription occurs in the absence of de novo viral protein synthesis or viral DNA replication and does not require the presence of VP16. Phase II, beginning 25 to 30 hours post treatment, is similar to normal productive infection, with a cascade of transcription initiated by the transcription of IE genes by VP16, and involving viral DNA synthesis and the production of infectious virus (119).

1.3 CELLULAR RESPONSES TO HSV-1 INFECTION

1.3.1 Innate Immunity

Intrinsic Immunity, the immediate response of the cell to HSV-1 infection where factors constitutively expressed directly repress incoming genomes, is reviewed in section 1.2.5. Innate immunity, in which cellular sensors detect viral infection and respond to it by initiating a reaction, such as activating a pathway, is discussed here.

Pattern Recognition Receptors

Toll-like Receptors (TLRs) are pattern recognition receptors (PRRs) that detect virus in cells and induce the expression of cytokines and chemokines. There are eleven identified mammalian TLRs, four of which recognize HSV-1. TLR-7 can activate NF- κ B, JNK, and p38 (120). Detection by TLR-2 can lead to the secretion of IL-6 and IL-12, possibly through the activation of NF- κ B. TLR-9 can detect CpG motifs in the viral genome and can upregulate cytokines and activate IFN- α (121, 122). TLR-3 can detect dsRNA and activate IFN- β , IL-6, NF- κ B, and IRF3 expression. Underscoring the importance of this PRR is evidence that mutations in TLR-3 make patients more susceptible to herpes simplex encephalitis (123, 124).

Other PRRs that detect viral infection include Protein Kinase R (PKR) and 2'—5' oligoadenylate synthetase (OAS). Both of these PRRs detect dsRNA. PKR can inhibit protein synthesis by phosphorylating eIF-2 α (125). OAS activates RNase L to degrade mRNA (126, 127).

IFI16 and cGas

HSV-1 DNA is sensed in the nucleus by IFI16, a DNA sensor that associates with viral DNA by 2 hours post infection (29, 122). IFI16 is required to induce IFN- β in HSV-1 infected cells and can detect incoming virus prior to viral transcription (128). Upon detecting viral DNA, IFI16 oligomerizes and activates STING, which activates TBK-1 phosphorylation. TBK1 can then phosphorylate IRF3, allowing IRF3 to dimerize, translocate to the nucleus, and promote IFN- β expression. cGAS can also bind to DNA, synthesize cGAMP and result in the expression of IFN- β . Although cGAS is normally found in the cytoplasm of cells, it is required for IFN- β expression during HSV-1 infection. Recent work has found a role for cGAS in stabilizing IFI16, potentially explaining how a cytosolic sensor can aid in the detection of nuclear DNA (129).

1.3.2 Adaptive Immunity

Antibodies against HSV-1 typically recognize antigens such as envelope glycoproteins, tegument proteins, or capsid proteins. Antibodies can typically be detected in the serum of infected patients (130). Antibodies to HSV-1 may play a protective role against infection, and antibodies to HSV-2 may hinder the transfer of HSV-2 to neonates (131). The T-cell response to HSV consists of T-cells primed by dendritic cells that recognize HSV infection via TLR2 and TLR9 (121).

1.3.3 Viral Evasion of Immune Responses

VHS and ICP27

Host protein synthesis is rapidly inhibited after the onset of infection—within one hour post infection (132). The tegument protein virion host shutoff protein (vhs) is an endoribonuclease

that degrades both cellular and viral mRNA to mediate a decrease in host cell transcription (133). VHS has been shown to play a significant role in the inhibition of the IFN I response to viral infection. Cells infected with vhs-null mutants produce more type I IFNs than cells infected with wild type HSV-1 (134–136). Recently, evidence has shown that vhs may also have a role in suppressing stress granule formation, reducing the presence of dsRNA in the cytoplasm of infected cells (137, 138). In addition to vhs, the IE protein ICP27 reduces the amount of translation of cellular genes by inhibiting mRNA splicing (139).

ICP0

The HSV-1 IE protein ICP0 is an E3 ubiquitin ligase that targets many cellular proteins for proteasomal degradation. ICP0 can counteract many cellular intrinsic antiviral defenses, including the repression of viral genomes by PML nuclear bodies and DNA-PKcs, as described in section 1.2.5. In addition to dispersing ND10 bodies, ICP0 also targets other restrictive cellular proteins that associate with viral genomes, including the cellular E3 ligases RNF8 and RNF168, for degradation (140). RNF8 and RNF 168 are involved in antiviral DNA damage response along with other cellular proteins targeted by ICP0 (140). Furthermore, there is evidence that the actions of ICP0 result in an inhibition of IRF3-mediated ISG induction in response to HSV-1 infection (141), possibly due to its role in the degradation of IFI16 (128, 142).

Inhibition of Specific Pathways

While vhs, ICP27 and ICP0 have far-reaching effects on general interferon responses and intrinsic immunity, some viral proteins have been implicated in the evasion of specific immune

response pathways. The tegument protein UL37 deamidates cGas, preventing it from catalyzing cGAMP synthesis and activating an innate immune response (143). Viral protein ICP34.5 inhibits STING signaling and PKR, preventing the activation of TBK1 (144). The viral protein US11 blocks OAS activation, preventing the cell from detecting dsRNA (145). Viral protein US3 can hyperphosphorylate IRF3, inhibiting its dimerization and translocation to the nucleus, thereby preventing IFN beta production (146).

Adaptive Immune Evasion

HSV-1 evades the T-cell response by preventing antigen presentation and modulation of apoptosis. The IE protein ICP47 inhibits peptide loading into MHC class I while the viral glycoprotein gB diverts MHC class II molecules to the exosome pathway (147–149). HSV-1 modulates apoptosis by inhibiting T-cell mediated apoptosis in infected non-immune cells and inciting apoptosis in infected T-cells (150, 151). Furthermore, HSV-1 glycoprotein heterodimer gE/gI can directly antagonize immune detection by binding antibodies in order to block complement activation and antibody-dependent cellular cytotoxicity (152). Viral glycoprotein gC can also block complement activation by binding complement component C3b (153).

1.4 HSV-1 AND TRANSCRIPTION

1.4.1 Viral Proteins Involved in Transcription

ICP4

The IE protein ICP4 is the main viral transcription regulator of HSV-1. As discussed in section 1.2.6, ICP4 is required for the expression of HSV-1 E and L genes (154). ICP4 forms a dimer that binds to DNA, and upon binding can multimerize, increasing affinity to the DNA (155, 156). ICP4 can also bind to a plethora of cellular proteins. ICP4 binds to transcription factors including TFIID, TAF1 subunit, TBP, and components of mediator, recruiting them to viral genomes (29, 58, 154, 157, 158). Through the recruitment of these transcription factors, ICP4 stabilizes the transcription initiation machinery on viral promoters (159, 160). The binding of ICP4 to viral gene promoters precedes the recruitment of TBP and RNA pol II to E and L gene promoters (161). Conversely, ICP4 also has a role in repressing IE gene transcription. It is hypothesized that ICP4 represses genes with an specially positioned ICP4 binding site (IE genes) by binding and interacting with TFIID and TFIIB in a way that prevents transcriptional activation (162). ICP4 is also required for the recruitment of factors such as cdk9 and ERCC3 (a component of TFIIH) (29).

ICP27

The IE protein ICP27 has many functions that affect viral and cellular gene expression. ICP27 inhibits splicing of cellular mRNAs by interacting with splicing factors such as Spliceosome-Associated Protein 145 (SAP145) and the kinase SRPK1 (163, 164). Through these interactions, ICP27 can alter the phosphorylation of a family of non-snRNP splicing proteins (SRs) (164,

165). Under normal conditions these proteins are involved in spliceosome assembly, and during HSV-1 infection alteration by ICP27 results in the incomplete assembly of the spliceosome and the inhibition of splicing (166).

ICP27 has been shown to promote the transcription of E and L genes through multiple actions, and mutants exhibit reduced viral DNA synthesis (167). ICP27 may increase 3' processing at the poly(A) sites of late genes (168). ICP27 has also been found to interact with ICP8 and RNA pol II (169, 170). ICP27 is also required for the nuclear export of viral mRNAs to the cytoplasm. ICP27 interacts with viral mRNA and TAP/NXF1, a major cellular mRNA nuclear export receptor (171, 172).

ICP22

The IE protein ICP22 has been implicated in the promotion of L gene transcription (78, 173–175). ICP22 is a 68-kDa peptide that contains two nuclear localization signals and localizes to the nucleus in both infected and transfected cells (176). ICP22 can be post translationally modified, with evidence of phosphorylation by the viral protein UL13 and nucleotidylation by the cellular protein Caesin Kinase 2 (CK2) (177–179). In addition, ICP22 is predicted to have intrinsically disordered domains. Extensive modification and intrinsically disordered domains support ICP22 having a complex role in infection and interaction with many proteins (180). ICP22 associates with HSV-1 DNA during productive infection (181).

Interestingly, the gene that encodes ICP22, *US1*, contains a second gene, *US1.5*. The protein produced by this gene would be a truncated form of ICP22, beginning around what would be the 90th residue of the full-length ICP22 protein. Although this truncated form of ICP22 may play a role in some of the functions attributed to full-length ICP22, not much work

has been done to clarify this point (182). The truncated protein will not be specifically discussed in this study.

ICP22 mutants produce infectious virus in Vero, HeLa, and HEp-2 cell lines. Conversely, ICP22 mutant virus is attenuated *in vivo* and in fibroblast cells such as MRC5 cells (78, 173, 183, 184). The reason for the cell-type dependent permissivity of these mutants has not been determined. Studies have shown that during infection of restrictive cells with an ICP22 mutant the expression of certain late genes is reduced (78, 79, 173, 175, 185). The mechanism by which ICP22 promotes the accumulation of late gene transcripts has yet to be determined. ICP22 mutant virions are also abnormally assembled, likely due to the fact that late genes produce structural and virion proteins (186). There is also evidence that ICP22 is important for the establishment of latency and reactivation from latency (184, 186).

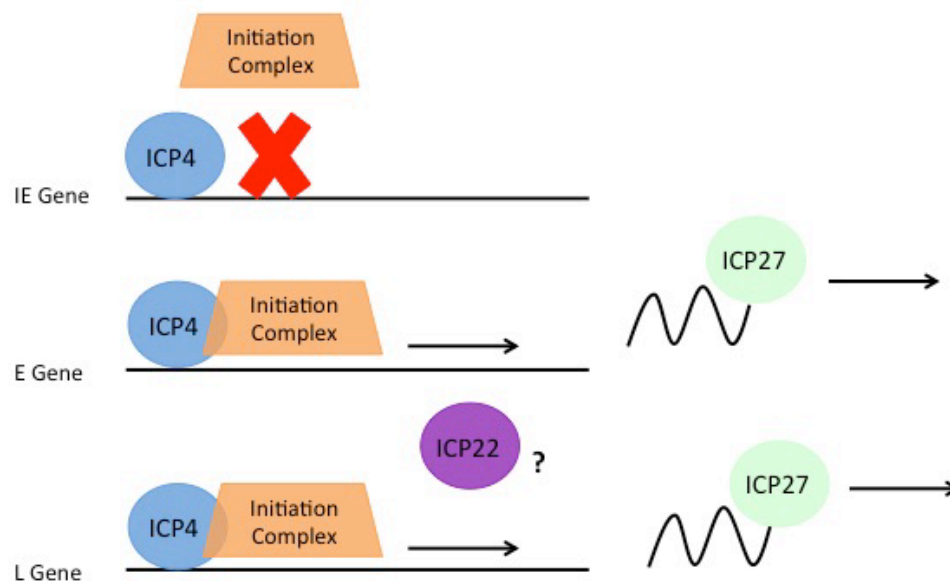


Figure 4. The roles of ICP4, ICP27, and ICP22 in HSV-1 transcription. ICP4 represses the transcription of IE genes and promotes the transcription of E and L genes. ICP22 plays a role in the transcription of L genes, promoting their transcription in some way. ICP27 is required for the export of viral mRNA.

1.4.2 Cellular Transcription Factors Associated with Viral DNA

In addition to cellular factors described in the previous sections with roles in viral productive infection that have been investigated, a number of other cellular transcription factors have been found to associate with viral DNA throughout infection. The cellular transcription factors related to the investigations in chapters 2 and 3 are reviewed here.

RNA pol II

RNA pol II is required for the transcription of viral genes. It is recruited to viral promoters by viral transcription regulators and associated with viral genomes throughout infection (29, 66, 85, 161, 181). Studies have also shown that RNA pol II is almost completely depleted from cellular genes during HSV-1 infection (187, 188), raising the question of how RNA pol II is recruited to, and maintained on, viral genes at the expense of cellular genes.

The C-terminal domain (CTD) of the large subunit of RNA pol II is composed of tandem repeats of a conserved consensus sequence that is modified throughout the regulated transcription of cellular genes (189). Differences in phosphorylation allow different proteins and complexes to dock on the CTD and promote different stages of transcription. The conserved CTD sequence contains five residues that can be phosphorylated, and all seven can be modified in other ways (190). This section will focus on the role of the phosphorylation of serine-5 (ser-5P) and serine-2 (ser-2P), as these are the most studied and relevant for the studies presented.

Unphosphorylated RNA pol II is recruited to gene promoters after which ser-5 is phosphorylated by TFIIH subunit cdk7 in a mediator-dependent manner (191). Ser-5P is required for the recruitment of the 5' capping enzymes (192). After transcription initiation, RNA pol II pauses at promoter-proximal regions. At this point RNA pol II is associated with negative

elongation factor (NELF) and DRB-sensitivity-inducing factor (DSIF). P-TEFb is then recruited to the paused ser-5P RNA pol II and the cdk9 subunit phosphorylates ser-2 and DSIF. The addition of ser-2P allows for the release of paused RNA pol II and also provides a docking site for chromatin modifiers, splicing factors, capping enzymes, and poly-adenylation machinery (193, 194). Ser-2P of RNA pol II is therefore associated with efficient elongation and termination of transcripts.

The phosphorylation of RNA pol II is altered during infection with HSV-1. Whereas the hyperphosphorylated ser-5P/ser-2P form is abundant in uninfected cell lysates, starting around 3 hours post infection this form disappears and is replaced by an abundance of ser-5P RNA pol II (195). ICP22 is required for the emergence of the ser-5P RNA pol II, but not for the disappearance of the ser-5P/ser-2P form (185). There is evidence that UL13 may enhance the reduction in ser-5P, possibly through its role in modifying ICP22 (174). The mechanism behind how the ser-5P/ser-2P form disappears upon infection and how transcription proceeds in the absence of ser-2P RNA pol II is unknown.

CDK9

Cdk9 is the component of P-TEFb responsible for the phosphorylation of ser-2 of RNA pol II. The role of cdk9 in HSV-1 infection, when ser-2P is absent, is unresolved. There are studies that propose that cdk9 is required for HSV-1 infection based on inhibitor assays (196, 197). Conversely, there are studies that propose that ICP22 binds to cdk9 to inhibit its activity (198, 199). Cdk9 is associated with HSV-1 DNA throughout infection and is not recruited to viral DNA in the absence of the viral IE protein ICP4 (29). The role of cdk9 in HSV-1 is still undefined.

FACT Complex

The FACT (facilitates chromatin transcription) complex is a histone chaperone that facilitates transcription elongation through nucleosomes (200). The FACT complex is a heterodimer of the proteins Spt16 and SSRP1(201). Although the FACT complex was originally found to promote transcription elongation by RNA pol II, the FACT complex can also facilitate transcription elongation by RNA polymerases I and III (202, 203). The FACT complex facilitates transcription elongation by destabilizing the interaction between H2A-H2B dimers and H3-H4 tetramers by removing a H2A-H2B dimer from the nucleosome ahead of RNA pol II (204, 205). The FACT complex also likely plays a role in reassembling nucleosomes on DNA after transcription (204, 206, 207). Spt16 has been implicated in the redeposition of the original H3-H4 dimers evicted during transcription elongation, maintaining histone modification patterns on actively transcribed genes (208). Additionally, the FACT complex may act as a specific exchange factor of histone H2A.X (209).

The FACT complex also has roles in regulating chromatin to allow for replication, DNA recombination, and DNA repair. The FACT complex has been found to bind to DNA polymerase α and DNA replication elongation complexes in yeast (210). The FACT complex also interacts with elongation factors such as Spt4-Spt5, Spt6, and Paf1 complex in yeast (211). The human FACT complex has also been found to have a physical and functional interaction with MCM helicase, contributing to the initiation of DNA replication (212). SSRP1 has a role in recombination-mediated DNA damage, while Spt16 is required for transcriptional restart after UV-induced DNA damage (213, 214). The FACT complex has also been found to interact with PARP1 and RPA, proteins involved in DNA damage repair processes such as homologous recombination (215)(216).

The FACT complex has been found to associate with HSV-1 genomes throughout infection (29, 85, 181). Furthermore, the FACT complex has been found to be an important factor in the infectious cycle of other Herpesviruses. SSRP1 is involved in the latent replication of KSHV and in the efficient transcription of CMV immediate early genes (217, 218).

1.5 RATIONALE

Herpes Simplex Virus 1 (HSV-1) is a common infection of the oral and genital mucosa. There is interest both in designing new therapeutics to treat HSV-1 infections and in using HSV-1 as a vector to design treatments for other ailments. Throughout the productive lifecycle of HSV-1 the virus interacts with and hijacks cellular proteins and in doing so alters numerous cellular pathways. Understanding how viral proteins regulate viral and cellular processes and also how cellular proteins are redirected by HSV-1 is important to developing treatments and understanding the effects of a potential vector.

In Chapter 2 we will determine how the FACT complex is recruited to viral DNA and elucidate the FACT complex's role in viral infection.

In Chapter 3 we will reveal the contribution of ICP22 to the repression of cellular transcription during productive HSV-1 infection.

2.0 A HERPESVIRAL IMMEDIATE EARLY PROTEIN PROMOTES TRANSCRIPTION ELONGATION OF VIRAL TRANSCRIPTS

The following study was published June 13, 2017 in mBio. “A Herpesviral Immediate Early Protein Promotes Transcription Elongation of Viral Transcripts” by Hannah L. Fox, Jill A. Dembowski, and Neal A. DeLuca. Copyright © 2017, American Society for Microbiology. Reproduced here under the terms of the Creative Commons CC BY license.

2.1 PROJECT SUMMARY

Herpes simplex virus 1 (HSV-1) genes are transcribed by cellular RNA polymerase II (RNA Pol II). While four viral immediate early proteins (ICP4, ICP0, ICP27, and ICP22) function in some capacity in viral transcription, the mechanism by which ICP22 functions remains unclear. We observed that the FACT complex (comprised of SSRP1 and Spt16) was relocalized in infected cells as a function of ICP22. ICP22 was also required for the association of FACT and the transcription elongation factors SPT5 and SPT6 with viral genomes. We further demonstrated that the FACT complex interacts with ICP22 throughout infection. We therefore hypothesized that ICP22 recruits cellular transcription elongation factors to viral genomes for efficient transcription elongation of viral genes. We reevaluated the phenotype of an ICP22 mutant virus by determining the abundance of all viral mRNAs throughout infection by transcriptome

sequencing (RNA-seq). The accumulation of almost all viral mRNAs late in infection was reduced compared to the wild type, regardless of kinetic class. Using chromatin immunoprecipitation sequencing (ChIP-seq), we mapped the location of RNA Pol II on viral genes and found that RNA Pol II levels on the bodies of viral genes were reduced in the ICP22 mutant compared to wild-type virus. In contrast, the association of RNA Pol II with transcription start sites in the mutant was not reduced. Taken together, our results indicate that ICP22 plays a role in recruiting elongation factors like the FACT complex to the HSV-1 genome to allow for efficient viral transcription elongation late in viral infection and ultimately infectious virion production.

2.2 IMPORTANCE

HSV-1 interacts with many cellular proteins throughout productive infection. Here, we demonstrate the interaction of a viral protein, ICP22, with a subset of cellular proteins known to be involved in transcription elongation. We determined that ICP22 is required to recruit the FACT complex and other transcription elongation factors to viral genomes and that in the absence of ICP22 viral transcription is globally reduced late in productive infection, due to an elongation defect. This insight defines a fundamental role of ICP22 in HSV-1 infection and elucidates the involvement of cellular factors in HSV-1 transcription.

2.3 INTRODUCTION

During productive infection, herpes simplex virus 1 (HSV-1) transcription is complexly regulated by both viral and cellular factors. Genes are transcribed by cellular RNA polymerase II (RNA Pol II) (219) in an ordered cascade with four classes: immediate early (α), early (β), leaky late (γ_1), and true late (γ_2) genes (61, 220). Four of the five immediate early proteins, ICP4, ICP0, ICP27, and ICP22, are involved in the regulation of viral transcription. ICP4 is a transcription factor that recruits cellular complexes, including TFIID and mediator, to viral genes to enhance transcription initiation and can also function to repress transcription of some viral genes (73, 221, 222). ICP0 is an E3 ubiquitin ligase (223) and plays a role in counteracting the repression of incoming viral genomes by host factors (224, 225) and abrogating the interferon response to infection (141, 226). ICP27 is involved in the nuclear export of viral mRNAs and has a role in recruiting RNA Pol II to viral genes (170, 227, 228). The immediate early protein ICP22 has been implicated in the promotion of late gene transcription, but the mechanism by which it functions is unknown (78, 173–175).

ICP22 is a 420-amino-acid protein that is extensively posttranslationally modified, including phosphorylation by the viral protein UL13 (177, 178, 229). ICP22 is required for efficient productive infection *in vivo* and in fibroblast cells such as MRC5 cells. ICP22 mutants are notably less restricted in Vero, HeLa, and HEp-2 cell lines (78, 173, 183, 184). Studies of ICP22 mutants in restrictive cells have shown that in the absence of ICP22 the expression of certain late genes is reduced (78, 79, 173, 175, 185). Additionally, virions produced by an ICP22 mutant virus are abnormally assembled, possibly due to reduced levels of the products of the late genes that encode virion assembly and structural proteins (186).

ICP22 expression is associated with two effects on the cellular environment that may be

related to its role in productive viral infection: the modification of RNA Pol II and the formation of VICE (virus-induced chaperone-enriched) domains (182). In uninfected cells, the C-terminal domain (CTD) of RNA Pol II is sequentially phosphorylated during cellular gene transcription. These modifications follow a pattern in which hypophosphorylated RNA Pol II is recruited to promoters and is then phosphorylated on serine-5 (Ser-5) of the CTD around the start of transcription initiation. As RNA Pol II proceeds along the gene, serine-2 (Ser-2) of the CTD is phosphorylated and Ser-5 phosphorylation gradually decreases toward the 3' end of the gene. Phosphorylation of Ser-2 of the CTD is typically associated with the ability of RNA Pol II to overcome promoter-proximal pausing and facilitate elongation (190, 230). During infection with HSV-1, the CTD of RNA Pol II is uniquely modified: RNA Pol II exhibiting both Ser-5 and Ser-2 phosphorylation of the CTD is rapidly decreased in the cell, and RNA Pol II exhibiting only Ser-5 phosphorylation of the CTD accumulates. The alteration of CTD modifications requires ICP22 and has been hypothesized to affect viral transcription in some way (185, 195, 231, 232). VICE domains have been shown to sequester host protein chaperones (233) and to accumulate nascent proteins during HSV-1 infection (234).

Our laboratory recently developed a modified iPOND (isolation of proteins on nascent DNA) protocol to purify viral genomes and analyze proteins associated with these genomes during productive infection (85, 181). Many viral and cellular proteins were found to be associated with viral genomes, including ICP22 and a number of cellular proteins involved in transcription. Some transcription factors identified by this method have been previously shown to interact with ICP4, such as TFIID and mediator (58). The FACT transcription elongation complex was one of the most abundant protein complexes identified and was found to relocate to viral replication compartments. The mechanism underlying this recruitment has not been

determined (85, 181).

Preliminary experiments with viruses deficient for one or more immediate early proteins suggested that ICP22 may be involved in the recruitment of the FACT complex to viral genomes. To elucidate the role of ICP22 in HSV-1 productive infection, we undertook to further characterize the phenotype of an ICP22 mutant. In this study, we purified wild-type (wt) and ICP22 mutant viral genomes from infected cells to determine which proteins require ICP22 in order to associate with viral DNA. We found that the amounts of FACT complex subunits SSRP1 and Spt16 were substantially reduced on ICP22 mutant genomes. The FACT complex was originally identified for its role in transcription elongation through nucleosomes (200). We therefore sought to characterize the role of ICP22 in the recruitment of the FACT complex to viral genomes during productive HSV-1 infection and to investigate the transcriptional defects that occur in the absence of ICP22. We demonstrate that ICP22 physically interacts with the FACT complex and is essential for FACT complex and transcription elongation factor recruitment to viral DNA. Furthermore, in the absence of ICP22, RNA Pol II association with gene bodies was reduced while the association with transcription start site regions of viral genes was not affected. Taken together, our results indicate that ICP22 plays a role in recruiting elongation factors, including the FACT complex, to viral genomes to allow for efficient transcription elongation of viral genes.

2.4 RESULTS

Previously, our lab has reported that the FACT complex is abundant on HSV-1 genomes during viral replication (181). Additionally, SSRP1 is redistributed within the nucleus upon infection in

KOS-infected cells compared to mock-infected cells, although Spt16 and SSRP1 protein levels are not altered by infection (181) (**Fig. 5**). In order to determine if any of the viral immediate early proteins were responsible for this redistribution and recruitment of the FACT complex to viral genomes, we used immunofluorescence to assay SSRP1 redistribution in cells infected with a panel of viruses deficient for one or more immediate early proteins (**Table S1**). We determined that SSRP1 was not redistributed upon infection with viruses deficient for ICP22. We therefore hypothesized that ICP22 may play a role in recruiting the FACT complex to viral genomes and that, by defining the relationship between ICP22 and the FACT complex, we could elucidate the role of ICP22 in productive infection.

Virus	Genes Mutated (ICP)	SSRP1 Relocalization
d109	4, 27, 22 , 47, 0	NO
d106	4, 27, 22 , 47	NO
d95	4, 27, 22	NO
d92	4, 27	YES
d96	4, 22	NO
DMP	27, 22	NO
d120	4	YES
5dl1.2	27	YES
d99	0	YES
n199	22	NO

Table S1. Analysis of SSRP1 localization in IE Mutants. Vero cells were infected with viruses defective in one or more HSV-1 immediate early protein. Infected cells were fixed and stained 3 hours post infection. Images were analyzed to determine whether SSRP1 reorganized into puncta (YES) or remained mostly in the nucleolus (NO). Virus references are included in Materials and Methods.

Redistribution of FACT complex subunit SSRP1 requires ICP22 expression. Immunofluorescence was used to further characterize the redistribution of the FACT complex relative to viral genomes as a function of ICP22. Vero cells were infected with either

wild-type virus (KOS) or ICP22 mutant virus (n199), and cultures were incubated with the nucleoside analogue in the medium from 4 to 6 h postinfection (hpi). 5-Ethynyl-2'-deoxycytidine (EdC) labeling of replicating viral genomes enables tagging of viral DNA with Alexa Fluor via click chemistry (85, 181). The cultures were fixed at 6 hpi, labeled viral genomes were tagged with Alexa Fluor, and SSRP1 was probed via indirect immunofluorescence. In mock-infected cells, SSRP1 was concentrated in the nucleolus (**Fig. 6A**), where it is known to play a role in RNA polymerase I-mediated transcription of rRNA (203). In KOS-infected cells, SSRP1 colocalized with viral genomes in replication compartments in addition to gathering in foci around viral replication compartments. In n199-infected cells, SSRP1 did not relocate and was excluded from viral replication compartments.

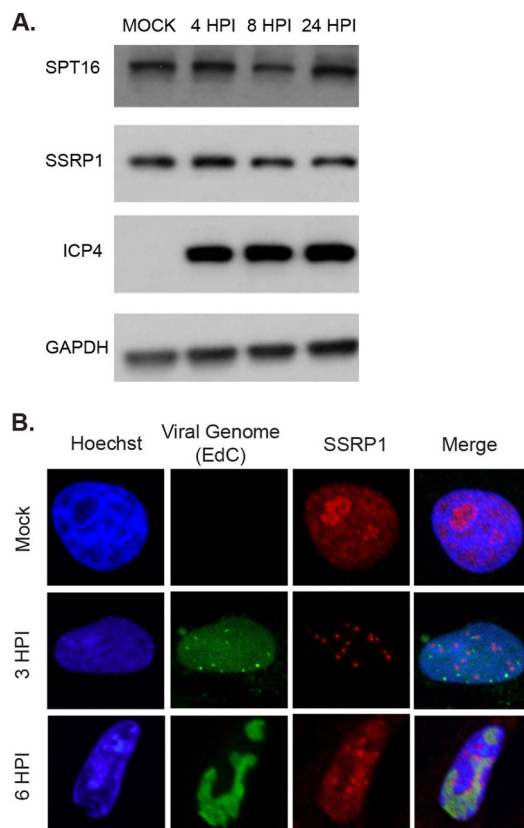


Figure 5. Abundance of FACT complex proteins and localization of SSRP1 in infected cells. (A) MRC5 cells were infected with wild-type (KOS) virus at an MOI of 10. Cell lysates were collected at the indicated hours postinfection. Cell lysates were run on a 10% SDS-PAGE gel and probed with antibodies against Spt16, SSRP1, ICP4, and GAPDH. (B) Vero cells were infected with unlabeled KOS, or KOS prelabeled with 10 μ M EdC, at an MOI of 10. Cells infected with prelabeled KOS were fixed at 3 hpi. Cells infected with unlabeled KOS were incubated in medium containing 10 μ M EdC starting at 4 hpi and then fixed at 6 hpi. Fixed cells were stained with Hoechst stain (blue) and reacted with Alexa Fluor azide as described in Materials and Methods to visualize viral DNA (green). SSRP1 was detected by immunofluorescence (red).

Because ICP22 is an immediate early protein, we also used immunofluorescence to visualize the localization of SSRP1 early in infection. Vero cells were infected with KOS or n199, fixed at 3 hpi, and probed with antibodies specific for ICP4 and SSRP1. ICP4 was used to represent the location of viral DNA because it has been shown to colocalize with viral genomes throughout infection (181). In cells infected with KOS, SSRP1 was redistributed into discrete foci in the nucleus by 3 hpi (**Fig. 6B**), and these foci were distinct from the majority of ICP4-containing foci. In cells infected with the ICP22 mutant n199, SSRP1 remained concentrated in

the nucleolus, resembling mock-infected cells. We additionally observed that in cells infected with viral mutants that express ICP22 but do not express ICP4 or other combinations of immediate early proteins (**Table S1**), SSRP1 is redistributed similarly to SSRP1 in wild-type-infected cells. The redistribution of SSRP1 in infected cells is therefore a specific function of ICP22. These data suggest that ICP22 affects the redistribution of SSRP1 in the cell throughout infection.

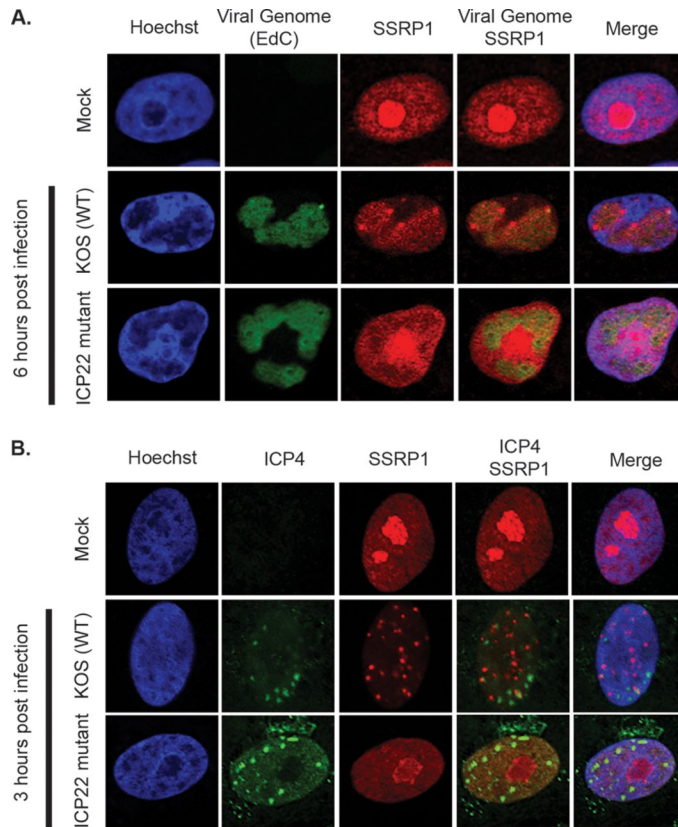


Figure 6. Abundance of FACT complex proteins and localization of SSRP1 in infected cells. (A) MRC5 cells were infected with wild-type (KOS) virus at an MOI of 10. Cell lysates were collected at the indicated hours postinfection. Cell lysates were run on a 10% SDS-PAGE gel and probed with antibodies against Spt16, SSRP1, ICP4, and GAPDH. (B) Vero cells were infected with unlabeled KOS, or KOS prelabeled with 10 μ M EdC, at an MOI of 10. Cells infected with prelabeled KOS were fixed at 3 hpi. Cells infected with unlabeled KOS were incubated in medium containing 10 μ M EdC starting at 4 hpi and then fixed at 6 hpi. Fixed cells were stained with Hoechst stain (blue) and reacted with Alexa Fluor azide as described in Materials and Methods to visualize viral DNA (green). SSRP1 was detected by immunofluorescence (red).

ICP22 is necessary for the association of elongation factors with viral genomes.In order to determine how ICP22 affects the association of the FACT complex and other viral and cellular factors with the viral genome, we purified replicating KOS and n199 viral DNA, along with associated proteins, from infected cells as previously described (85, 181). We infected MRC5 cells at a multiplicity of infection (MOI) of 10 PFU/cell with either n199 or KOS and incubated cells in the presence of EdC from 4 to 6 hpi to label replicating viral DNA. Infected cultures without EdC added to the medium served as a negative control. At 6 hpi, nuclei were harvested and EdC-labeled viral DNA was covalently tagged with biotin via click chemistry. Genomes were then purified on streptavidin beads, and associated proteins were identified by mass spectrometry. Many viral and cellular proteins were enriched on both the KOS and n199 viral genomes relative to unlabeled negative controls (**Table S2**).

The association of most viral and cellular proteins with viral genomes was comparable between KOS and n199 as indicated by the similar recoveries of spectral counts (SpC) (**Table 1**). Levels of viral DNA binding proteins ICP4, UL42, and ICP8 were very similar between KOS and n199 EdC-labeled samples. Furthermore, the viral replication proteins UL9 (origin binding protein) and UL30 (DNA polymerase), as well as cellular DNA repair proteins, were present at similar levels on KOS and n199 genomes. Transcription initiation factors were found to be present at similar levels on KOS and n199 genomes or were slightly increased on n199 genomes. Interestingly, the levels of a subset of cellular transcription factors were selectively reduced on n199 genomes relative to KOS genomes. Most striking was the relative deficiency of a number of cellular transcription elongation factors. Specifically, there was a reduction in Spt5, Spt6, and FACT complex members Spt16 and SSRP1, as well as a modest reduction in the levels of RNA

Pol II subunits. Both Spt16 and SSRP1 levels were reduced more than 20-fold, providing evidence that ICP22 plays a role in recruiting the FACT complex to replicating viral genomes.

	Replicate 1		Replicate 2	
	KOS	n199	KOS	n199
Viral Proteins				
ICP4	689	696	834	916
ICP22	60	2	63	3
ICP8	592	539	972	910
UL9	199	218	255	341
UL30	213	229	456	507
UL42	369	255	411	320
Cellular Repair Proteins				
XRCC5 (KU80)	89	88	101	130
XRCC6 (KU70)	62	66	55	65
RNA Polymerase II Subunits				
PolrA	35	17	102	82
PolrB	19	6	47	42
Transcription Elongation Factors				
Spt16	57	4	78	7
SSRP1	43	0	29	0
Spt5	0	0	32	10
Spt6	12	0	41	2
Cdk9	5	9	6	11
Transcription Initiation Factors				
TBP (TFIID)	0	0	6	5
TAF1 (TFIID)	0	0	5	12
TAF6 (TFIID)	0	10	2	8
ERCC2 (TFIIH)	6	13	8	13
ERCC3 (TFIIH)	3	13	12	32
MED12 (Mediator)	13	20	43	62
MED14 (Mediator)	8	26	41	92
MED23 (Mediator)	23	50	60	112
MED 24 (Mediator)	15	23	25	52

Table 1. Proteins Associated with Viral Genomes. MRC5 cells were infected with wild-type (KOS) or an ICP22 mutant (n199) at an MOI of 10 and were incubated with EdC-containing medium or negative-control medium (no EdC) from 4 to 6 hpi. At 6 hpi, nuclei were harvested and the DNA was purified as described in Materials and Methods. Proteins eluted from genomes were analyzed by mass spectrometry. Values indicate spectral counts determined by mass spectrometry.

ICP22 interacts with FACT complex members SSRP1 and Spt16. Results thus far demonstrated that ICP22 is required for the relocalization of the FACT complex in infected cells and its association with viral genomes. In order to determine if these observations involve an interaction between ICP22 and FACT complex components, we constructed a virus expressing

N-terminally FLAG-tagged ICP22 in a KOS background (ICP22-FLAG) to use in immunofluorescence and coimmunoprecipitation experiments.

As in wt virus-infected cells, SSRP1 was relocalized from the pattern seen in mock-infected cells to discrete nuclear foci in ICP22-FLAG-infected cells (**Fig. 7A**). Furthermore, FLAG-tagged ICP22 colocalized with SSRP1. The number of SSRP1 foci in ICP22-FLAG-infected cells in **Fig. 7A** is fewer than the number of foci seen in **Fig. 6B** in wt virus-infected cells. While this may indicate that the relocalization is delayed in ICP22-FLAG-infected cells, we believe that this is most likely due to cell-to-cell variation.

We therefore immunoprecipitated ICP22-FLAG to determine if FACT complex members physically interact with ICP22. We infected cells with ICP22-FLAG, lysed cells at 3 or 6 hpi, and immunoprecipitated ICP22-FLAG and associated proteins with anti-FLAG antibody-coated magnetic beads. Mock-, KOS-, and n199-infected cells were used as controls. Proteins were eluted from the beads with 3×FLAG peptide and subjected to Western blot analysis. Both of the FACT complex subunits, SSRP1 and Spt16, coprecipitated with ICP22. Treatment of purified complexes prior to immunoprecipitation with Benzonase, a nuclease that degrades all forms of DNA and RNA, did not disrupt the interactions between ICP22 and FACT complex members, indicating that the presence of nucleic acid in the sample did not contribute to coprecipitation (**Fig. 7B**). Taken together, these data indicate that ICP22 interacts with complex members either directly or as part of a complex.

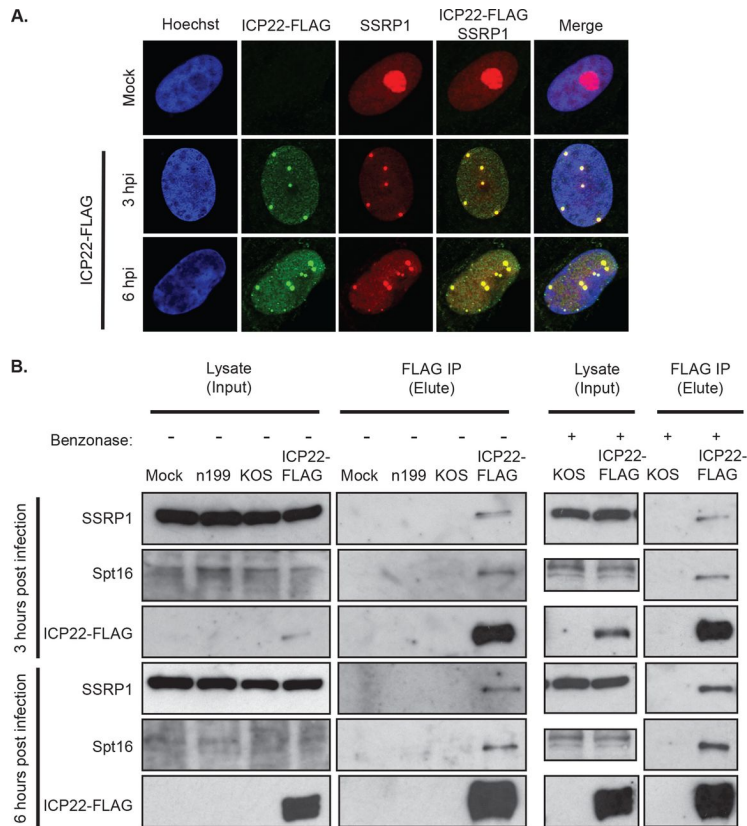


Figure 7. Interaction of ICP22 and the FACT complex in infected cells. (A) Localization of SSRP1 and ICP22-FLAG in infected cells. Vero cells were infected with virus expressing FLAG-tagged ICP22 protein (ICP22-FLAG) at an MOI of 10. Cells were fixed at 3 and 6 hpi and stained with Hoechst stain (blue). ICP22-FLAG (green) and SSRP1 (red) were detected by immunofluorescence. Single cells shown are representative of cells in two independent replicates. (B) Isolation of FLAG-tagged ICP22 and associated proteins in infected cells. MRC5 cells were infected with ICP22-FLAG at an MOI of 10. Cells were harvested at 3 or 6 hpi. Lysates were treated with (+) or without (-) Benzodiazepine and bound to anti-FLAG beads. Proteins bound to the beads were eluted with 3 \times FLAG peptide and run on an SDS-PAGE gel. Gels were immunoblotted with anti-SSRP1, anti-Spt16, and anti-FLAG.

Viral mRNA and genome abundance in ICP22 mutant-infected cells. To further investigate the significance of the interaction between the FACT complex and ICP22, we reevaluated the gene expression and DNA replication phenotypes of n199 (235) using more sensitive approaches. It has previously been reported that the rate of viral DNA replication of ICP22 mutant virus is similar to that of wild-type virus based on hybridization assays (78, 185, 235). To obtain a more quantitative account of viral DNA replication in the absence of ICP22, we used quantitative PCR to measure the number of viral genomes over time. We carried out infections in

MRC5 cells, which are known to be restrictive to ICP22 mutant virus. One million cells were infected at an MOI of 5, and DNA was harvested throughout the productive viral replication cycle. DNA replication rates did not differ significantly between KOS and n199, indicating that ICP22 likely does not play a significant role in viral DNA replication (**Fig. 8A**). We also assessed infectious virus production throughout infection using plaque assays. We observed an almost 20-fold reduction in infectious virus produced from n199-infected cells compared to KOS-infected cells (**Fig. 8A**). These data suggest that although the n199 genome efficiently replicates, it has some defect or defects that prevent infectious virion production.

Previously, a reduction in late gene expression has been presumed to contribute to the reduced infectivity associated with n199. We therefore quantified viral mRNA abundance throughout infection to identify transcriptional defects with n199. Transcriptome sequencing (RNA-seq) was used to measure viral transcript levels at different times after infection with wt (KOS) or ICP22 mutant (n199) virus. RNA-seq data indicated that although late gene mRNA accumulation was indeed impaired in n199 as reported, the expression of viral genes in other classes was also affected (**Fig. 8B**). Reduced gene expression appears to depend more on the time postinfection than on the kinetic class of a particular gene. Specifically, the expression of viral genes that are increasingly expressed after 6 hpi in KOS was reduced in n199 compared to KOS, regardless of kinetic class. These genes include those previously identified to be less expressed in ICP22 mutant-infected cells, such as RL1, which codes for ICP0; US11 (175); UL48, which codes for VP16; UL44, which codes for gC; and UL36 (185). We also observed decreased expression of genes such as β genes UL9, UL42, UL50, and UL52; γ 1 genes US6 and UL19; and γ 2 genes UL45, UL35, and UL38, among others.

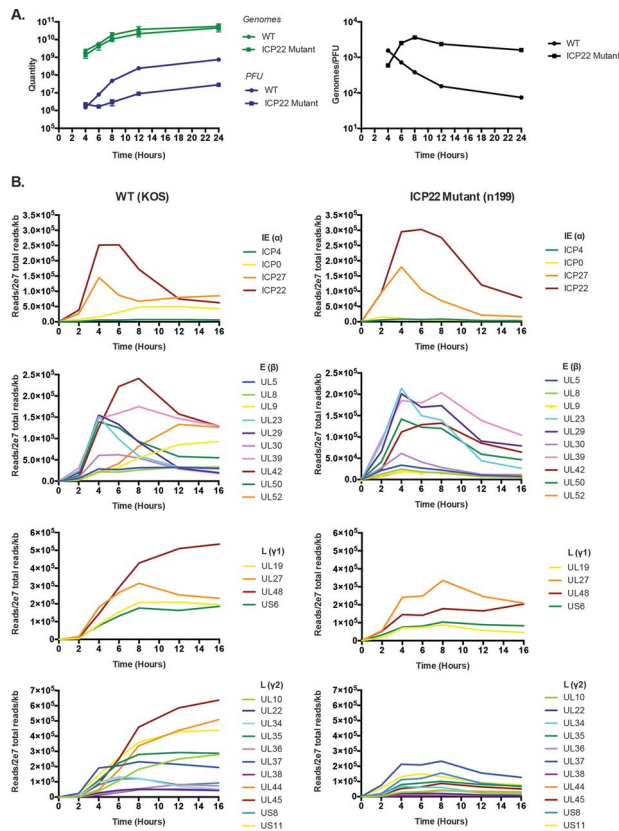


Figure 8. Replication and gene expression of an ICP22 mutant. (A) DNA replication and viral replication of ICP22 mutant n199 compared to wild-type KOS. MRC5 cells were infected and assayed at 4, 6, 8, 12, and 24 hpi. DNA replication was determined by quantitative PCR (genomes). Points are averages from 2 experiments; error bars represent standard deviations. Viral replication was determined by plaque assay (PFU). Points are averages from 2 experiments; error bars represent standard deviations. (B) RNA-seq analysis of select genes in all kinetic classes in ICP22 mutant (n199) compared to wild type (KOS). MRC5 cells were infected at an MOI of 5 and assayed at 2, 4, 6, 8, 12, and 16 hpi. Viral transcripts were quantified by RNA-seq as described in Materials and Methods.

Elongation of RNA polymerase transcription is impaired in an ICP22 mutant. In ICP22 mutant-infected cells, there was a global reduction in the abundance of all viral transcripts late in infection (**Fig. 8B**). Furthermore, the main difference in the recruitment of cellular proteins to the n199 genome relative to KOS appears to be limited to factors involved in transcription elongation (**Table 1**). In order to determine if the reduced abundance of viral transcripts in the absence of ICP22 could be due to a defect in transcription elongation, we mapped the distribution of RNA Pol II on KOS and n199 genomes by chromatin immunoprecipitation sequencing (ChIP-seq) at 6 hpi, a critical time for the observed phenotypes. MRC5 cells were

infected with either KOS or n199 and cross-linked at 6 hpi to capture the state of the genome at that time, and parallel immunoprecipitations were performed with antibodies specific for RNA Pol II and ICP4.

In wt HSV-1-infected cells, a unique form of RNA Pol II (Ili), in which the CTD is phosphorylated on Ser-5, accumulates. Additionally, reduction in the unphosphorylated form (IIa) and even greater reduction in the hyperphosphorylated form (Ilo), in which the CTD is phosphorylated on both Ser-5 and Ser-2, are observed (195, 231). In ICP22 mutant virus-infected cells, the Ili form does not accumulate and there is greater abundance of the IIa form than in KOS-infected cells (185). We performed parallel immunoprecipitations with the two RNA Pol II antibodies 8WG16 and 4H8. 8WG16 recognizes both the IIa and the HSV-specific Ili forms of RNA Pol II, both of which are present in HSV-1-infected cells (195). 4H8 recognizes Ser-5-modified RNA Pol II and should therefore recognize Ili. Additionally, 4H8 recognizes RNA Pol II with both Ser-5 and Ser-2 phosphorylated because Ser-2 phosphorylation does not inhibit binding of the antibody to its Ser-5 epitope and should therefore recognize Ilo (190). Although 8WG16 is more specific for IIa than 4H8, 4H8 can also recognize IIa. Both 8WG16 and 4H8 have been used in previous ChIP-seq studies to determine RNA Pol II levels on individual genes and have been found to detect similar patterns (236). Therefore, by using both 8WG16 and 4H8, we are able to detect all forms of RNA Pol II that have been identified to be present in KOS- and n199-infected cells (185).

Immunoprecipitated DNA was sequenced to determine the relative abundance of RNA Pol II and ICP4 across the viral genome. Consistent with results from the DNA replication assay (**Fig. 8A**), the total numbers of reads that map to the viral genome in the input samples were similar between KOS and n199 (**Fig. S1A**). Furthermore, consistent with mass spectrometry

analysis (**Table 1**), the numbers of reads that map to the viral genome in ICP4 ChIP samples were similar between KOS and n199 and the numbers of reads that map to the viral genome in the RNA Pol II ChIP samples were somewhat reduced for n199 relative to KOS (**Fig. S1A**).

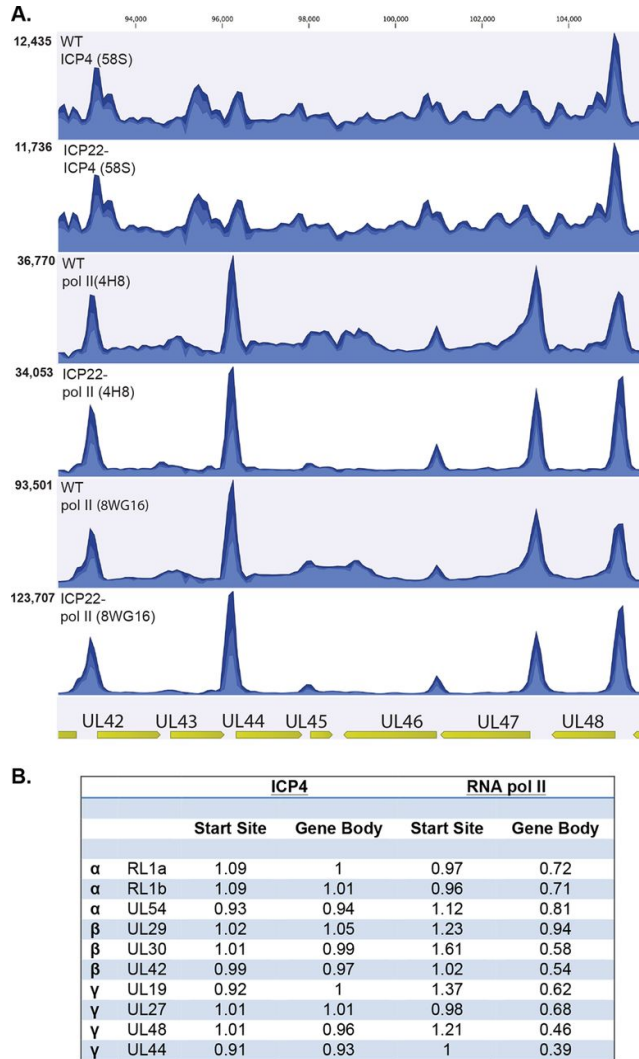


Figure 9. ChIP analysis of ICP4 and RNA Pol II on ICP22 mutant versus wt (KOS) genomes. MRC5 cells were infected with wild type (KOS) or ICP22 mutant (n199) at an MOI of 10. The infected cells were harvested and processed for ChIP-seq as described in Materials and Methods. (A) Mapped reads of the region between UL42 and UL48 on the genome for ChIP of ICP4, RNA Pol II (4H8), or RNA Pol II (8WG16) in n199- or KOS-infected cells. The maximums on the y axis are to the top left of each panel. Experiments were repeated twice, and the figure represents one data set. (B) Normalized ratio of n199 over KOS reads for start sites or gene bodies of representative IE, E, and L genes. The values used were averages from two experiments using the 4H8 antibody.

The reads mapping across the viral genome for ICP4 and RNA Pol II are shown in **Fig. S1B**. The distribution of ICP4 across the viral genome was unaffected by mutation of ICP22. The details of ICP4 binding to the viral genome will be published elsewhere. At this level of resolution, the difference between the RNA Pol II binding profiles of n199 and KOS is quite subtle. The peaks in the n199 samples appear to be better defined with a possible reduction in read numbers between the peaks compared to the KOS samples. 8WG16 and 4H8 antibodies produced read patterns similar to one another and reveal the same differences in binding patterns between KOS and n199.

A higher-resolution representation of **Fig. S1B** in the region between UL42 and UL48 is shown in **Fig. 9A**. These genes are all expressed at 6 hpi in KOS-infected cells and represent members of the early through true late classes of genes. The numbers of reads in the RNA Pol II ChIP at or near transcription start sites were similar between KOS and n199. However, the numbers of reads mapping beyond the start site, in the message bodies, appeared to be significantly reduced in n199 (**Fig. 9A**).

In order to compare the relative abundance of Pol II and ICP4 on the start sites and message bodies of n199 and KOS, we calculated the ratio of normalized n199 to KOS reads for individual gene mRNA start sites and gene bodies of select genes representative of different kinetic classes for both ICP4 and RNA Pol II (**Fig. 9B**). For the calculations, we used the data from the 4H8 antibody as this antibody should detect the forms of RNA Pol II involved in the transcription of viral genes in KOS- and n199-infected cells, including Ser-5-phosphorylated RNA Pol II and RNA Pol II phosphorylated on both Ser-5 and Ser-2 (Ilo). An example of the analysis is shown in **Fig. S2**. In the ICP4 immunoprecipitations, n199-over-KOS read ratios were consistently close to 1 for both gene start sites and gene bodies. On the other hand, the n199-

over-KOS read ratios for the RNA Pol II immunoprecipitations were reduced on n199 viral gene bodies compared to KOS, particularly for genes whose mRNA levels were reduced later in infection in the ICP22 mutant (**Fig. 8B**). The number of RNA Pol II reads on transcription start sites was not reduced in n199 relative to KOS, with more reads on the n199 start sites in some cases (**Fig. 9B**). This observation supports a model in which transcription elongation is reduced in the absence of ICP22; however, transcription initiation is unaffected.

2.5 SUPPLEMENTAL FIGURES

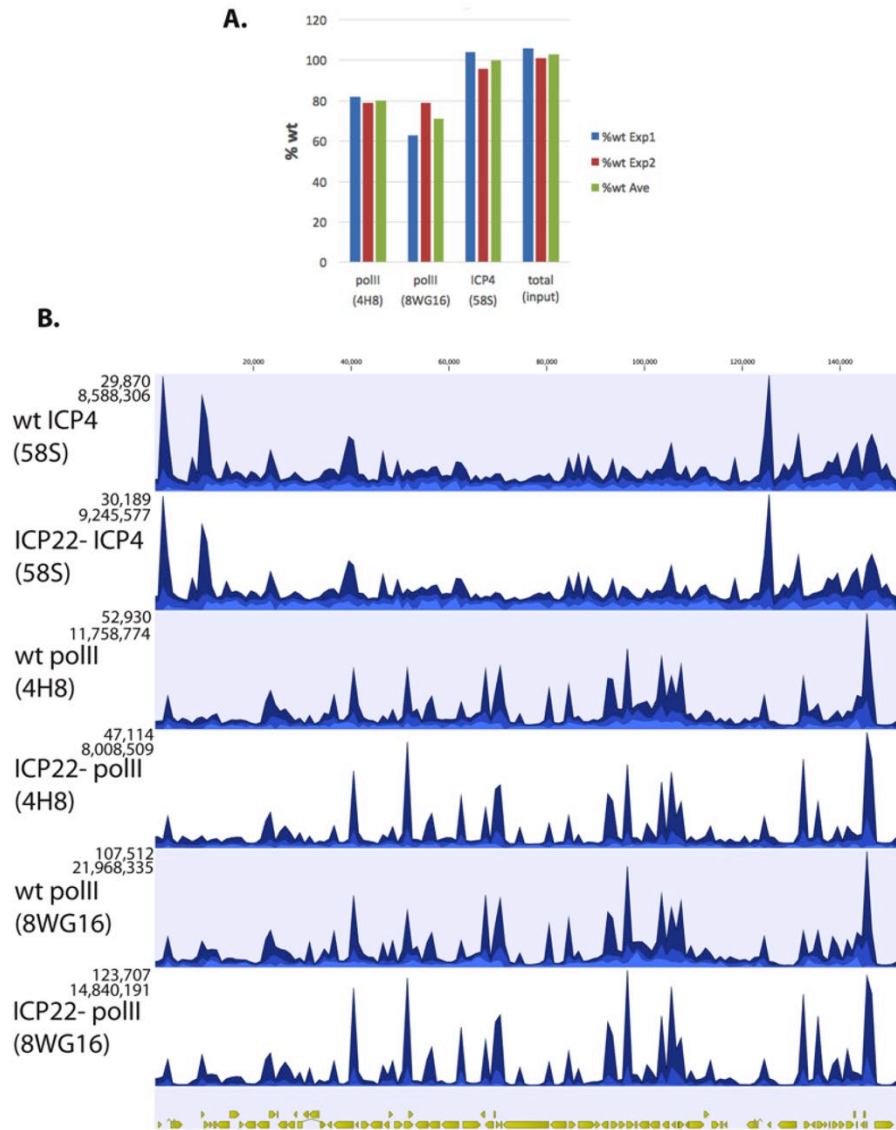


Figure S1. Total ChIPseq Reads. (A) Percent of reads from n199 samples over KOS samples. Data for each replicate and average of the two replicates. (B) Mapped reads on the HSV-1 genome for ChIP of ICP4, RNA Pol II 4H8, and RNA Pol II 8WG16 in n199 or KOS infected cells. The maximums on the y-axis are listed at the top of each panel. The number below is the total mapped reads.

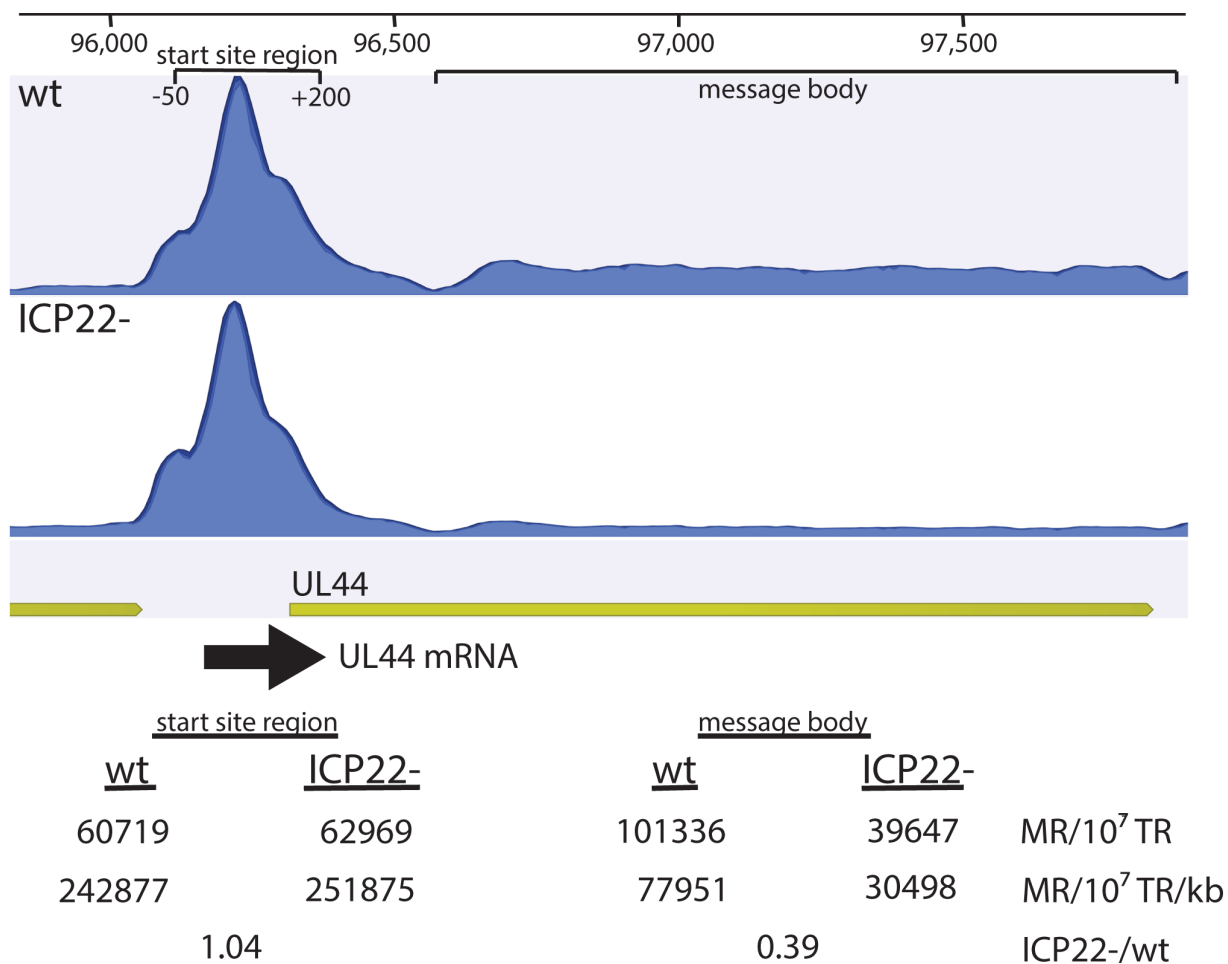


Figure S2. Calculation of Start Site and Gene Body Reads for Selected Genes. A schematic of how the numbers in Figure 4B were calculated using the gene UL44 as an example.

2.6 DISCUSSION

HSV-1 genes are transcribed by cellular RNA Pol II (219). However, the Pol II machinery of the cell is perturbed or augmented by viral gene products, resulting in the selective and efficient expression of viral genes. For example, the immediate early protein ICP4 plays an important role in the regulation of transcription initiation. ICP4 binds to viral DNA and interacts with crucial cellular proteins involved in transcription initiation such as transcription factors, TFIID, and mediator (58, 158), facilitating their recruitment to the viral genome. In this study, we describe findings that shed light on how the immediate early protein ICP22 affects RNA Pol II transcription of viral genes, thus promoting the production of viral progeny. Specifically, ICP22 interacts with the FACT complex and is responsible for the relocation of FACT in the nucleus and its recruitment to the viral genome along with two other transcription elongation factors, Spt5 and Spt6. In addition, while ICP22 was not required for wt levels of RNA Pol II on the transcription start sites of viral genes, the amount of RNA Pol II in the bodies of viral genes was significantly reduced in the absence of ICP22. These observations suggest that ICP22 promotes the elongation of RNA Pol II transcription of viral genes late after infection, likely through the recruitment of transcription elongation factors.

The FACT complex is a histone chaperone that is known to dissociate H2A-H2B dimers from nucleosomes to allow access to DNA by elongating RNA Pol II and other factors (204, 237). The FACT complex is involved in cellular transcription elongation in addition to other processes in the cell such as DNA replication (238, 239). In addition to displacing nucleosomes by dissociating H2A-H2B dimers, the FACT complex can also destabilize nucleosomes globally, contributing to the maintenance of a loose chromatin structure (206). The FACT complex may therefore contribute to the establishment and maintenance of the loosely associated chromatin

structure observed on HSV-1 genomes during viral DNA replication (240). Furthermore, the FACT complex has been found to be involved in the latent replication of Kaposi's sarcoma-associated herpesvirus and in the efficient transcription of human cytomegalovirus immediate early genes (217, 218). In addition to the FACT complex, we discovered a substantial decrease in the amount of Spt6 and Spt5 recruited to viral genomes in the absence of ICP22 (**Table 1**). All of these proteins have established roles in cellular transcription elongation and are known to interact with RNA Pol II and one another (241–244). If these factors are not recruited to paused RNA Pol II, transcription arrest can occur (190).

Previously, it was determined that late gene transcription, as well as the transcription of the immediate early gene that codes for ICP0, is attenuated in ICP22 mutants (78, 173, 175, 186). However, the connection between the genes affected was not elucidated. It has been suggested that a common sequence may be responsible for uniting those genes affected by ICP22 (182). We used RNA-seq to characterize the transcription of viral genes in the ICP22 mutant and observed a global decrease in viral mRNA abundance beginning at 4 to 6 hpi, after the onset of viral DNA replication. Late genes are affected more drastically and noticeably because they are typically upregulated 4 to 6 hpi. Immediate early and early genes that are typically expressed at consistent or rising levels at 4 to 6 hpi, including ICP0, are impacted by the absence of ICP22 in a manner similar to late genes (**Fig. 8B**). Consistent with previous observations (78, 185, 235) we did not find a defect in viral DNA replication in the n199 background (**Fig. 8A**).

The observation that transcription elongation factors are reduced on ICP22 mutant genomes suggests that transcription elongation may be responsible for the inefficient viral gene expression in ICP22 mutants. This hypothesis is further supported by the ChIP-seq data demonstrating a specific reduction in the amount of RNA Pol II on the bodies of viral genes in

the ICP22 mutant compared to wild-type virus, whereas that amount of RNA Pol II on their transcription start sites was relatively unaffected (**Fig. 9**).

The forms of RNA Pol II in HSV-infected cells are relevant to these observations. In uninfected cells, unphosphorylated RNA Pol II (IIa) is recruited to promoters, where it is phosphorylated on Ser-5 at the start of transcription initiation. Ser-2 phosphorylation of paused RNA Pol II results in the hyperphosphorylated form of RNA Pol II (IIo) (245). This form of RNA Pol II is closely associated with actively elongating cellular transcripts, and elongation factors such as Spt6 are known to bind to Ser-2-phosphorylated forms (246, 247). However, Ser-2-phosphorylated forms of Pol II are greatly depleted in wild-type and ICP22 mutant virus-infected cells (185, 195, 231, 232, 248). This raises the question of how transcription elongation occurs in infected cells with altered Pol II CTD phosphorylation.

The formation of the IIo form of RNA Pol II requires the action of cdk9. However, there is a lack of consensus on the role of phosphorylation of Ser-2 of the CTD by cdk9 in HSV-1 gene transcription. It has been postulated that ICP22 interacts with or recruits cdk9 in order to phosphorylate Ser-2 of the CTD (197, 249). There is evidence that ICP22 physically associates with cdk9 (197, 249) and that inhibition of kinases such as cdk9 can lead to decreased transcription during HSV-1 infection (196). Others have observed a rapid loss of RNA Pol II Ser-2 phosphorylation when ICP22 is expressed in cells either transiently or at later times during infection, indicating that Ser-2 phosphorylation by cdk9 may not be necessary for efficient viral transcription (79, 185, 231, 232). Moreover, it has been proposed that ICP22 interacts with cdk9 in order to inhibit it and prevent Ser-2 phosphorylation (198, 199). Supporting this is the observation that cellular transcripts are repressed during infection with HSV-1. Notably, this reduction in cellular transcription is enhanced late in infection (248). It may be that cdk9 and

Ser-2 phosphorylation is more important early in infection and that late after infection ICP22 is required to sustain transcription elongation. In addition, persisting small amounts of Ser-2 phosphorylation in Vero cells may be a possible explanation for the reduced requirement of ICP22 in Vero cells relative to human fibroblasts. The possible temporal involvement of cdk9 in HSV infection and the basis for the differential requirement for ICP22 in different cell types remain to be tested.

We propose that ICP22 facilitates the recruitment of Spt5, Spt6, and the FACT complex to transcribing RNA Pol II independently of the form of Pol II involved in elongation in uninfected cells. Evidence exists that ICP22 interacts with RNA Pol II (196). The recruitment of the elongation factors through these interactions would then facilitate the elongation of viral transcripts, possibly by ensuring a looser chromatin structure on viral gene bodies. The precise nature of these interactions, the role of chromatin, and the implications for cellular transcription are under investigation.

2.7 MATERIAL AND METHODS

Cells and viruses. Experiments were performed using MRC5 (human fetal lung) or Vero (African green monkey kidney) cells obtained from and propagated as recommended by ATCC. The viruses used in this study include wild-type KOS, 22/n199 (n199), and ICP22-FLAG. n199 is derived from wild-type KOS and contains a linker carrying stop codons in the US1 gene as has been previously described (235). ICP22-FLAG was generated using red-mediated recombination in a bacterial artificial chromosome (BAC) containing KOS DNA (250–252). A FLAG tag was inserted after the start codon of US1. Modified BAC constructs were then transfected into Vero

cells using Lipofectamine 2000 transfection reagent (Life Technologies, Inc.). Virus produced was harvested, plaque purified, and sequenced to verify FLAG tag insertion. Additional viruses used include d109, d106, d92, and d99 (53); d95, d96, and DMP (253); d120 (222); and 5dl1.2 (167).

Immunoblotting for protein abundance. MRC5 cells were infected at an MOI of 10 by incubation with KOS virus in Tricine-buffered saline (TBS) for 1 h before inoculum was removed and cells were rinsed with TBS. Cells were then incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for the indicated amount of time. Samples were rinsed with ice-cold TBS plus 0.1 mM tosyl-l-lysyl-chloromethane hydrochloride (TLCK) before being scraped into 2× SDS-PAGE sample buffer. Samples were boiled for 10 min before analysis. Samples were run on a 10% precast gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (Amersham), and probed with the following primary antibodies: rabbit anti-Spt16 H300 (Santa Cruz; 1:500), mouse anti-SSRP1 10D1 (BioLegend; 1:500), anti-ICP4 58S (1:500), and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) Ambion AM4300. After incubation with anti-rabbit–horseradish peroxidase (HRP) or anti-mouse–HRP secondary antibody (Promega), bands were visualized using ECL Prime Western blot detection reagent (Amersham).

Imaging of viral DNA and immunofluorescence. Vero cells (2×10^5) were plated on coverslips in 12-well dishes. Cells were infected at an MOI of 10 by incubation with virus in TBS for 1 h before inoculum was removed and cells were rinsed with TBS. Cells were then incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for the indicated amount of time. Labeling of replicating genomes was carried out as described previously (181). Briefly, EdC (10 μ M) was added to the medium of infected cells

at 4 hpi and fixed at 6 hpi. EdC-labeled DNA was then visualized by conjugating EdC to Alexa Fluor 488 azide using the Click-iT Alexa Fluor 488 imaging kit (Thermo Fisher). The immunofluorescence assay was carried out as described previously (181) with the following primary antibodies: rabbit anti-ICP4 N15 (1:500), mouse anti-SSRP1 10D1 (BioLegend; 1:200), and mouse anti-FLAG M2 (Sigma; 1:5,000). Secondary antibodies used included goat anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen; 1:500), goat anti-mouse conjugated to Alexa Fluor 594 (Invitrogen; 1:500), goat anti-mouse IgG2b conjugated to Alexa Fluor 594 (Invitrogen; 1:500), and goat anti-mouse IgG1 conjugated to Alexa Fluor 488 (Invitrogen; 1:500). Images were obtained using an Olympus FluoView FV1000 confocal microscope.

Isolation of viral genomes and associated proteins. Viral DNA isolation was carried out as described previously (85) with the following modifications. MRC5 cells were infected with KOS or n199 at an MOI of 10 and incubated at 37°C in DMEM containing 2% FBS for 4 h, at which time 10 μ M EdC was added to the medium. Infections carried out in the absence of EdC were used as negative controls. At 6 hpi, nuclei were harvested and processed as described above. Genome-associated proteins were analyzed by mass spectrometry as described previously (181). Mass spectrometry was carried out by MSbioworks, LLC, Ann Arbor, MI.

Coimmunoprecipitation and immunoblotting. MRC5 cells were grown to confluence in a 150-mm plate. Cells were infected at an MOI of 10 by incubation with indicated virus or mock infection in TBS for 1 h before the inoculum was removed and cells were rinsed with TBS. Cells were then incubated at 37°C in DMEM containing 2% FBS for the indicated amount of time. After incubation, cells were rinsed with ice-cold TBS plus 0.1 mM tosyl-l-lysyl-chloromethane hydrochloride (TLCK). Cells were then lysed at 4°C for 30 min in 1.5 ml lysis buffer (50 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1% Triton X-100, 1 mM EDTA, Roche

protease inhibitor cocktail). Lysates were then centrifuged at $1.8 \times 10^3 \times g$ for 10 min at 4°C, and supernatants were filtered through a 100-µm cell strainer (Corning). Indicated samples were incubated in 10 µl Benzonase (Novagen) at this step for 10 min. Samples were then combined 1:1 with binding buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM EDTA, Roche protease inhibitor cocktail) and added to anti-FLAG M2 magnetic beads (Sigma). Immunoprecipitation was carried out according to the manufacturer's protocol with a few modifications. Briefly, samples were bound to the anti-FLAG beads overnight and following a series of washes with Tris-buffered saline (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, Roche protease inhibitor cocktail). Bound proteins were eluted using the recommended concentration of 3×FLAG peptide (Sigma). Samples were combined 1:1 with 2× SDS-PAGE sample buffer and boiled for 10 min before analysis. Samples were run on a 7.5% precast gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (Amersham), and probed with the following primary antibodies: rabbit anti-Spt16 H300 (Santa Cruz; 1:500), mouse anti-SSRP1 10D1 (BioLegend; 1:500), and mouse anti-FLAG M2 (Sigma; 1:500). After incubation with anti-rabbit–HRP or anti-mouse–HRP secondary antibody (Promega), bands were visualized using ECL Prime Western blot detection reagent (Amersham).

Viral DNA quantification and growth curve. MRC5 cells (1×10^6) in 35-mm plates were infected with n199 or KOS at an MOI of 5. Cells were infected by incubation with indicated virus or mock infected in TBS for 1 h before removing inoculum and rinsing cells three times with warm TBS. Cells were then incubated at 37°C in DMEM containing 2% FBS for the indicated amount of time.

For DNA quantification, cells were lysed in 200 µl DNA lysis buffer (0.5% SDS, 100 mM NaCl, 400 µg/ml proteinase K), incubated at 37°C 4 h to overnight, incubated at 65°C

for 30 min, and diluted 1:1,000 in 1× TE (100 mM Tris, 10 mM EDTA). Quantitative real-time PCR was carried out as described previously (62). Genome quantity was determined based on the amplification of the HSV-1 TK gene using the following primers: TkdsF1 (5'-ACC CGC TTA ACA GCG TCA ACA-3') and TkdsR1 (5'-CCA AAG AGG TGC GGG AGT TT-3'). Standard curves were established using serially diluted purified KOS genomic DNA.

To assess infectious virus production, cells were harvested by scraping into incubation medium. Cells were then lysed by freeze-thaw and sonication, following which cells were pelleted and supernatant was isolated. Supernatant was serially diluted and used to infect Vero cell monolayers in 6-well plates. After infection, cells were incubated at 37°C in overlay medium (DMEM, 10 g/liter methylcellulose, 2% FBS). After 3 days, the medium was removed and cells were stained with 10 mg/ml crystal violet in 50% ethanol. PFU was determined as an average from two duplicate wells.

RNA-seq. MRC5 cells were seeded into 60-mm dishes at a density of 2×10^6 cells per dish, infected with n199 or KOS at an MOI of 10 PFU/cell, and incubated at 37°C. At the appropriate time postinfection, RNA was isolated using the Ambion RNaqueous-4 PCR kit using the included protocol. The isolated total RNA was quantified using an Agilent eukaryotic RNA Nano kit and a 2100 Bioanalyzer. One microgram of total RNA from each sample was used to create sequencing libraries using the TruSeq RNA sample preparation kit (Illumina). Individual samples each with unit barcodes were quantified using an Agilent DNA 7500 chip and a 2100 Bioanalyzer. Pooled multiplexed samples were sequenced at the Tufts University genomics facility. The demultiplexed sequence read files were mapped to the HSV strain KOS genome and quantified using CLC Genomics Workbench.

ChIP-seq. MRC5 cells were seeded into 600-cm² square tissue culture dishes at a density of 7×10^7 cells per dish, infected with n199 or KOS at an MOI of 10 PFU/cell, overlaid with 100 ml medium, and incubated at 37°C. At 6 hpi, 5 ml of 20% formaldehyde was added to the culture medium. The cultures were incubated at room temperature for 5 min, followed by the addition of 5 ml of 2.5 M glycine. All subsequent procedures were performed at 4°C unless otherwise stated. The cultures were then washed with TBS and scraped into 50 ml of FLB {5 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], pH 8.0, 85 mM KCl, 0.5% Igepal CA-630 [US Biological], Roche protease inhibitor cocktail}. The cells were then pelleted by low-speed centrifugation, resuspended in 5 ml FLB, and pelleted again. The cell pellet was resuspended in 1.2 ml RIPA buffer (1× phosphate-buffered saline [PBS], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Roche protease inhibitor cocktail) and sonicated for 6 intervals of 30 s each on ice with a Sonics Vibra-Cell VCX 130 sonicator equipped with a 3-mm microprobe. The sonicated material was centrifuged at $1.8 \times 10^3 \times g$ for 15 min. DNA was isolated from 50 µl of the supernatant as a total input control. The remainder was divided equally to use in immunoprecipitations using the ICP4 and RNA Pol II monoclonal antibodies, 58S and either 8WG16 (Abcam; ab817) or 4H8 (Abcam, Inc.; ab5408), respectively. Twenty-five microliters of monoclonal antibody was bound to 50 µl of Dynabeads M280 sheep anti-mouse IgG beads (Invitrogen) in 5% bovine serum albumin (BSA) in PBS overnight. The antibody-coated beads were extensively washed with 5% BSA in PBS and then added to the reserved sonicated extracts, which were incubated overnight. The immunoprecipitation mixtures were washed five times with LiCl wash buffer (100 mM Tris-HCl buffer, pH 7.5, 500 mM LiCl, 1.0% Igepal CA-630, 1.0% sodium deoxycholate) and once with TE. The beads were then resuspended in IP elution buffer (1.0% sodium dodecyl sulfate, 0.1 M NaHCO₃) and incubated at 65°C for

2 h, after which time the beads were removed and incubation of the supernatant was continued overnight at 65°C to reverse cross-links. The samples were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) twice and with chloroform-isoamyl alcohol (24:1) once and then purified using Qiagen PCR cleanup columns. The quantity of DNA in each sample was quantified using a Qubit 2.0 fluorometer (Invitrogen). Ten nanograms of each sample was used to create sequencing libraries using the NEBNext Ultra II DNA library preparation kit (New England Biolabs). Individual samples each with unit barcodes were quantified using an Agilent DNA 7500 chip and a 2100 Bioanalyzer. Pooled multiplexed samples were sequenced at the Tufts University genomics facility. The demultiplexed sequence read files were mapped to the HSV strain KOS genome and analyzed using CLC Genomics Workbench.

3.0 THE ROLE OF ICP22 IN THE INHIBITION OF CELLULAR TRANSCRIPTION DURING HSV-1 INFECTION

3.1 PROJECT SUMMARY

HSV-1 gene transcription requires viral proteins, cellular transcription factors, and RNA polymerase II. As viral transcripts accumulate during productive infection, cellular transcription is repressed, with viral transcripts exceeding cellular transcripts in infected cells by 6 hours post infection. Using RNA-seq we have determined that a virus in which ICP22 is mutated is not only inefficient at promoting the transcription of viral genes, but is also inefficient at repressing cellular transcription. In cells infected with wild type virus, cellular transcripts are substantially downregulated compared to mock infected cells at 6 hours post infection. Fewer cellular genes are downregulated in cells infected with ICP22 mutant virus compared to mock infected cells at 6 hours post infection. We observe this trend escalate throughout the course of infection. In order to determine how transcription shifts from cellular to viral genes we used ChIP-seq to determine the position and abundance of RNA polymerase II on genes at 6 hours post infection. We determined that levels of RNA polymerase II are reduced on cellular genes in wild type infected cells compared to mutant infected cells, suggesting that a lack of recruitment of RNA pol II away from cellular genes may be responsible for the reduced shift from viral to cellular transcription in cells infected with the ICP22 mutant. Additionally, we present evidence that the aberrant

phosphorylation of RNA pol II observed during HSV-1 infection is an important aspect of this strategy. Furthermore, we identified viral and cellular proteins that interact with ICP22 during infection that may be directly recruited by ICP22 to viral genomes.

3.2 IMPORTANCE

HSV-1 eludes cellular immune responses primarily by reducing cellular gene expression at the levels of both transcription and translation. The mechanisms by which HSV-1 accomplishes this while abundantly expressing viral genes are important to understanding HSV-1 pathogenesis. We demonstrated that the viral protein ICP22 usurps cellular proteins in order to promote viral gene transcription at the expense of cellular gene transcription, reducing the ability of cells to respond effectively to viral infection. We therefore present a viral mechanism that enhances viral infection by a direct action on viral production and a seemingly indirect action in cellular transcription, reflecting the intricate interactions between HSV-1 and its host.

3.3 INTRODUCTION

Herpes Simplex Virus (HSV-1) is a common human pathogen that primarily infects epithelial cells and establishes latency in sensory neurons (14). Productive viral infection causes extensive changes in the host cell nucleus, as HSV-1 uses many cellular factors to assist in viral transcription, replication, and assembly (181). In addition to usurping cellular factors and

pathways, HSV-1 also floods host cells with viral proteins of various functions, and suppresses the natural reaction of cells to foreign invaders.

HSV-1 has developed many well-characterized mechanisms to evade cellular responses to infection. Some viral proteins directly target antiviral and immune responses, and some more generally shut down cellular processes that the cell needs to mount a response. Viral tegument proteins are transported between the virion and envelope, allowing for their release upon viral entry into cells. The tegument protein VP22 targets the inflammasome (254). The tegument protein VP16 allows for the rapid transcription of viral Immediate Early (IE) genes (255). The IE protein ICP0 both blocks interferon-stimulated gene (ISG) induction (141) and degrades antiviral factors such as PML to allow for transcription of viral genes (39). Additionally, HSV-1 proteins contribute to a decrease in the abundance of cellular transcripts. The virion host shutoff protein (VHS), a tegument protein, degrades mRNA (256), and the IE protein ICP27 has a role in inhibiting the splicing of cellular mRNAs (139). The overall reduction in cellular transcription upon HSV infection has also been supported by the observations of reduced RNA polymerase II (RNA pol II) on cellular genes (187, 188) and deficient termination of cellular transcripts (257). The exact mechanism behind these observations has yet to be defined.

HSV-1 requires cellular RNA pol II, along with many other viral and cellular factors, to transcribe its genes. Viral transcription is temporally regulated, with genes classified in order of expression: immediate early (IE), early (E), and late (L) (61, 220). The IE proteins ICP4, ICP22, and ICP27 are especially important for the transcription of subsequent gene classes. ICP4 is required for the transcription of E and L genes. ICP4 binds to DNA, and interacts with transcription factors such as TFIID and mediator, recruiting them to viral genomes (58, 222). ICP27 is involved in viral mRNA nuclear export, and may also have roles in other stages of

transcription (170, 227). We have recently demonstrated that ICP22 interacts with, and/or is required for the recruitment of, transcription elongation factors like Spt6 and the FACT complex, promoting efficient viral transcript elongation (258).

Although viral gene transcription is similar to cellular gene transcription, there are some differences that could help explain why viral genes are abundantly transcribed at the expense of cellular genes. First, few viral mRNAs are spliced, making them less sensitive to the detrimental effects of ICP27 on splicing. Second, cellular transcription is regulated by the phosphorylation of the C-terminal domain (CTD) of RNA pol II. Serine-5 phosphorylation (ser-5P) is associated with transcription initiation, and subsequent serine-2 phosphorylation (ser-2P) is associated with transcription elongation (230, 259). During HSV-1 infection, levels of RNA pol II with both ser-5 and ser-2 phosphorylation (ser-5P/ser2P) are reduced in favor of RNA pol II with only ser-5P, a switch associated with ICP22 (185, 195, 231, 232). This switch in phosphorylation may benefit the virus by reducing the transcription elongation of cellular transcripts. Viral transcripts, to which ICP22 can recruit elongation factors, may be efficiently elongated by RNA pol II with only ser-5P, the most abundant form during infection.

Recent papers have used sensitive techniques such as 4SU-sequencing and Pro-seq to explore the effects of HSV-1 infection on cellular transcription termination and RNA pol II position on cellular genes, respectively (188, 260). Although these studies have characterized the state of cellular transcription in cells infected with HSV-1, the complex effects of HSV IE proteins and other elements of infection on transcription have limited conclusions pertaining to mechanism. Here, we use RNA-seq and ChIP-seq of cells infected with ICP22 mutant virus to determine the contribution of ICP22 to the downregulation of cellular transcripts upon infection. We confirm that HSV-1 infection globally downregulates cellular transcription, and,

additionally, we suggest that alterations to cellular transcription are a result of the actions of ICP22 in addition to the accumulation of viral genomes throughout infection and other viral mechanisms. Specifically, we propose that ICP22 affects transcription at the level of elongation, recruiting co-transcriptional factors to viral genes. Viral genes can therefore be transcribed by RNA pol II with only ser-5P, and benefit from the change in levels of modified RNA pol II during infection. Additionally, increased viral transcription taking place on viral genomes due to the efficient initiation and elongation of viral transcripts results in higher levels of RNA pol II on viral genomes, leaving less available for the transcription of cellular genes.

3.4 RESULTS

Cellular Transcription in the Presence and Absence of ICP22

To investigate the effect of ICP22 on cellular transcription throughout HSV infection, we quantified viral and cellular mRNA accumulation using transcriptome sequencing (RNA-seq). MRC5 cells were infected at an MOI of 5 and RNA was harvested at 2, 4, 6, 8, and 12 hours post infection (hpi). In cells infected with wild type virus (KOS), as viral transcription increases and the viral life cycle progresses, cellular transcription decreases. Viral transcripts surpass cellular transcripts in WT virus (KOS) infected cells by 6 hpi (Fig. 10A).

Throughout infection WT virus (KOS) and ICP22 mutant virus (n199) DNA replicates at similar rates (258), allowing us to explore the effect of ICP22 on cellular transcription that is unrelated to viral genome replication. We found that during infection with an ICP22 mutant virus (n199), viral transcripts increased and cellular transcripts decreased at a similar rate as in WT

(KOS) infected cells until about 4 hpi. By 6 hpi, while viral transcripts eclipse cellular transcripts and continued to accumulate in cells infected with WT virus (KOS), this did not occur in cells infected with ICP22 mutant virus (n199) (Fig 10A). Exploring this difference gave us the opportunity to elucidate the role of ICP22 on cellular transcription and genome accessibility.

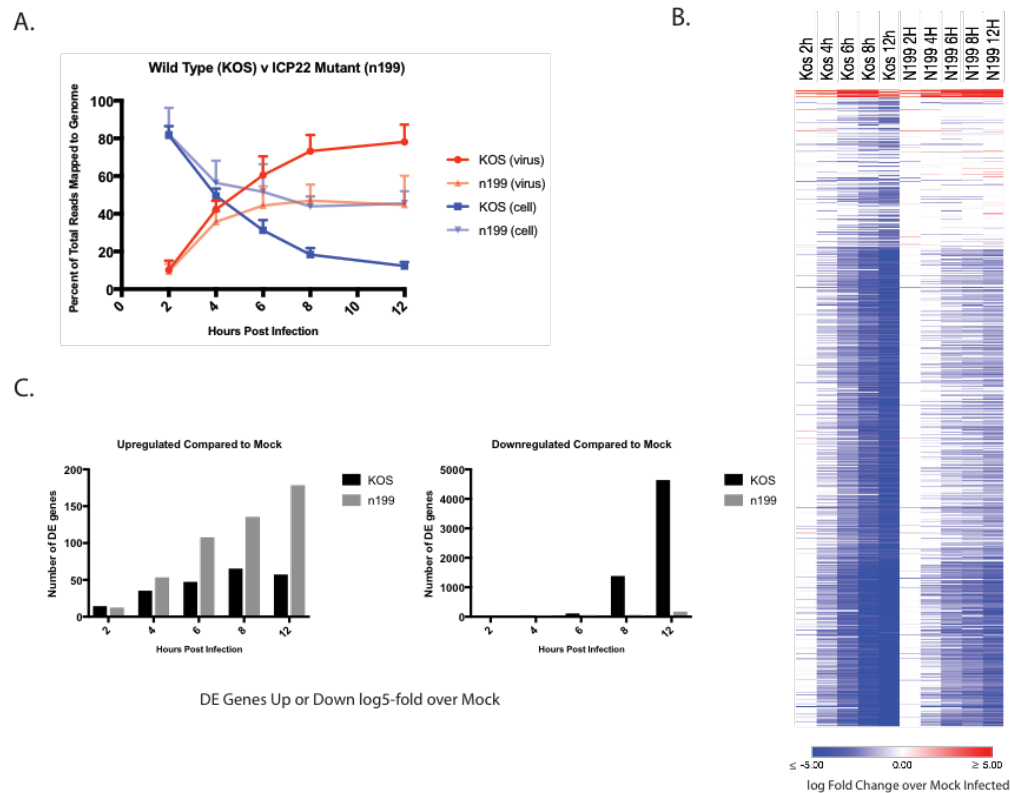


Figure 10. Changes to gene expression in cells infected with HSV-1 with or without ICP22 over the course of productive infection. MRC5 cells were infected at an MOI of 5 with wild type (KOS) virus, ICP22 mutant virus (n199), or TBS (Mock). RNA was harvested at 2, 4, 6, 8, and 12hpi. RNA-seq analysis, followed by differential expression analysis, was performed as described in the methods. (A) Percent of total reads in the sample at each time point that map to either the cellular or viral genome. Data represents 3 biological replicates with standard deviation. (B) Heat map of significantly differentially expressed cellular genes ($p < 0.05$). EdgeR was used to calculate the logFC of reads mapped to each gene in virus (KOS or n199) infected cells compared to mock infected. Data is based on 3 replicates. (C) Genes expressed logFC of 5 or more (Upregulated) compared to mock and logFC of -5 fold or less (Downregulated) compared between wild type (KOS) and ICP22 mutant (ICP22).

In order to better understand the scope of the effect of infection on cellular gene transcription, we used concurrent transcriptome sequencing of mock-infected cells to calculate differential expression of individual cellular genes between mock-infected samples and samples

infected with WT virus (KOS) or ICP22 mutant virus (n199). Including mock-infected samples allowed us to measure expression in infected cells relative to normal expression of cellular genes. Comparing the differential expression of these genes in cells infected with WT (KOS) or ICP22 mutant (n199) virus allowed us to determine which changes in cellular transcription are due to the presence of ICP22. EdgeR was used to calculate which genes were significantly differentially expressed due to infection with either WT (KOS) or ICP22 mutant (n199) virus at any point during infection. Comparing these genes across samples in a heat map confirmed what we observed with overall transcript levels and indicated that there is a global downregulation of many cellular genes during WT (KOS) infection. Many cellular genes were significantly downregulated throughout infection with WT (KOS), and very few were upregulated (Fig 10B). The number of genes downregulated increased throughout infection and the degree to which they were downregulated also increased. In cells infected with ICP22 mutant virus (n199), fewer genes were downregulated over time than in cells infected with WT (KOS) virus and these genes were less robustly downregulated as well (Fig. 10B). The cellular genes that were most differently expressed in cells infected with WT (KOS) or ICP22 mutant (n199) virus (defined as log fold change greater than 5 or less than -5) followed a similar pattern to genes differentially expressed to a lesser extent. Throughout infection with ICP22 mutant virus, more genes were robustly upregulated than throughout infection with WT (KOS) virus. More strikingly, thousands more genes were robustly downregulated throughout infection in cells infected with WT virus (KOS) rather than ICP22 mutant virus (n199) (Fig. 10C).

Alteration of Cellular Pathways During HSV-1 Infection

Although the downregulation of cellular transcripts by HSV-1 infection appears to affect transcription in general as opposed to targeting specific cellular genes, some of the pathways affected by these transcriptional changes may be ones that would otherwise respond to viral infection, thereby allowing HSV-1 to hinder a cellular immune response. Due to the rapid nature with which wild type HSV-1 shuts down host immune responses, Immediate Early (IE) mutants such as d109 (which expresses no IE proteins) have been used to illuminate the host immune response that is mounted by cells and immediately counteracted by the actions of IE proteins (141). Including d109 in our transcriptome analysis allowed us to elucidate the effect of ICP22 on the expression of cellular genes involved in the innate response to HSV-1 infection.

MRC-5 cells were infected with wild type (KOS), ICP22 mutant (n199), mock (TBS) or IE mutant (d109) virus at an MOI of 10 and assayed at 2 hpi and 6 hpi. In contrast to infection with both WT (KOS) and ICP22 mutant (n199), we found many cellular transcripts that were statistically and substantially upregulated in d109 infected cells at both 2hpi and 6hpi compared to mock-infected cells (Fig. 11A, 12A). By 6 hpi, 1378 cellular transcripts were uniquely upregulated in cells infected with d109 as opposed to those infected with WT (KOS) or ICP22 mutant (n199) virus (Fig. 12A). Integrative Pathway Analysis (IPA) performed on these genes revealed an upregulation of gene sets involved in immune response pathways as well as other cellular signaling pathways (Fig. 12B).

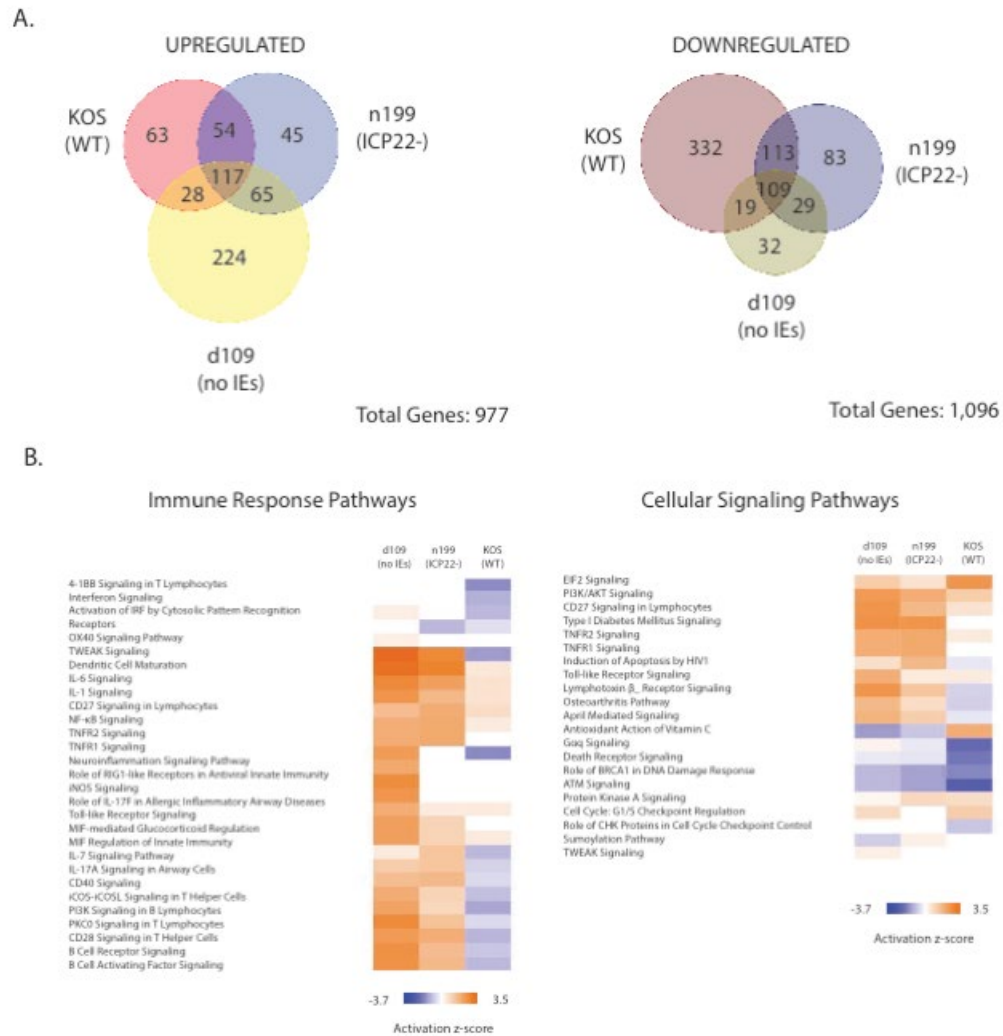


Figure 11. Differences in pathway regulation due to HSV-1 infection at 2 hours post infection. MRC5 cells were infected at an MOI of 5 with wild type (KOS) virus, ICP22 mutant virus (n199), IE mutant virus (d109), or TBS (Mock). RNA was harvested at 2 hpi. RNA-seq analysis, followed by differential expression analysis, was performed as described in the methods. EdgeR was used to calculate the logFC of reads mapped to genes in virus (KOS, n199, or d109) infected cells compared to mock infected. Data is based on 3 replicates. (A) Venn Diagrams of cellular genes significantly differentially expressed ($p < 0.05$) logFC of 1 or more (Upregulated) compared to mock and logFC of -1 fold or less (Downregulated) in cells infected with KOS, n199, or d109. (B) Ingenuity Pathway Core Analysis was performed on all 3 conditions: infection with KOS, n199, or d109. Cellular genes significantly differentially expressed ($p < 0.05$) were analyzed based on their logFC compared to Mock and p-value from DE analysis on 3 replicates in order to determine canonical pathways affected in each condition. Comparison analysis between the core analyses demonstrated differences in the degree of activation of different pathways between the conditions and are represented by differences in z-score in the heat maps. Selected pathways involved in the immune response and cellular signaling that significantly differed among conditions are clustered and shown in the heat map.

At 2 hpi, prior to viral DNA replication, IE proteins are already having an effect on the accumulation of cellular transcripts. 117 genes and 109 genes were upregulated or downregulated, respectively, by all three viruses (KOS, n199, and d109) compared to mock (Fig. 11A). These changes may be due to cellular responses to viral DNA and tegument proteins. Pathways that are activated in cells infected with all three viruses compared to mock infected include IL-6 Signaling, IL-1 signaling and NF κ B signaling, although most of these are activated to a greater degree in both d109 and n199 infected cells than in KOS infected cells. These pathways are considered to be antiviral and demonstrate a host response to HSV-1 infection being activated and then reduced due to the actions of IE proteins including ICP22. Additionally, the majority of immune response pathways affected were repressed in KOS infected cells even at early times in infection, including both Interferon Signaling and Activation of IRF by Cytosolic Pattern Recognition (Fig. 11B).

One pathway that was activated more in KOS infected cells compared to d109 or n199 infected cells is EIF2 signaling (Fig. 11B). Although many cellular processes are not required to complete the viral life cycle or would possibly hinder the viral lifecycle, the virus may be specifically activating the EIF2 pathway in order to encourage rapid synthesis of viral proteins.

By 6 hpi a similar number of genes were upregulated by KOS and n199, as at 2 hpi, but many more are upregulated in cells infected with d109. Many thousands of genes were downregulated by KOS, of which over half of were also downregulated by n199 (Fig. 12A). Even pathways previously activated at 2 hpi, such as IL-6, IL-1 and NF- κ B, were highly repressed in cells infected with KOS or n199, although they remained at least somewhat activated in d109 (Fig. 12B). Alternatively, almost all genes downregulated by n199 were also downregulated by KOS (Fig. 12A). The downregulation of the shared genes is most likely due to

features that wild type and ICP22 mutant viruses share such as viral genome replication, ICP0, and ICP4 expression.

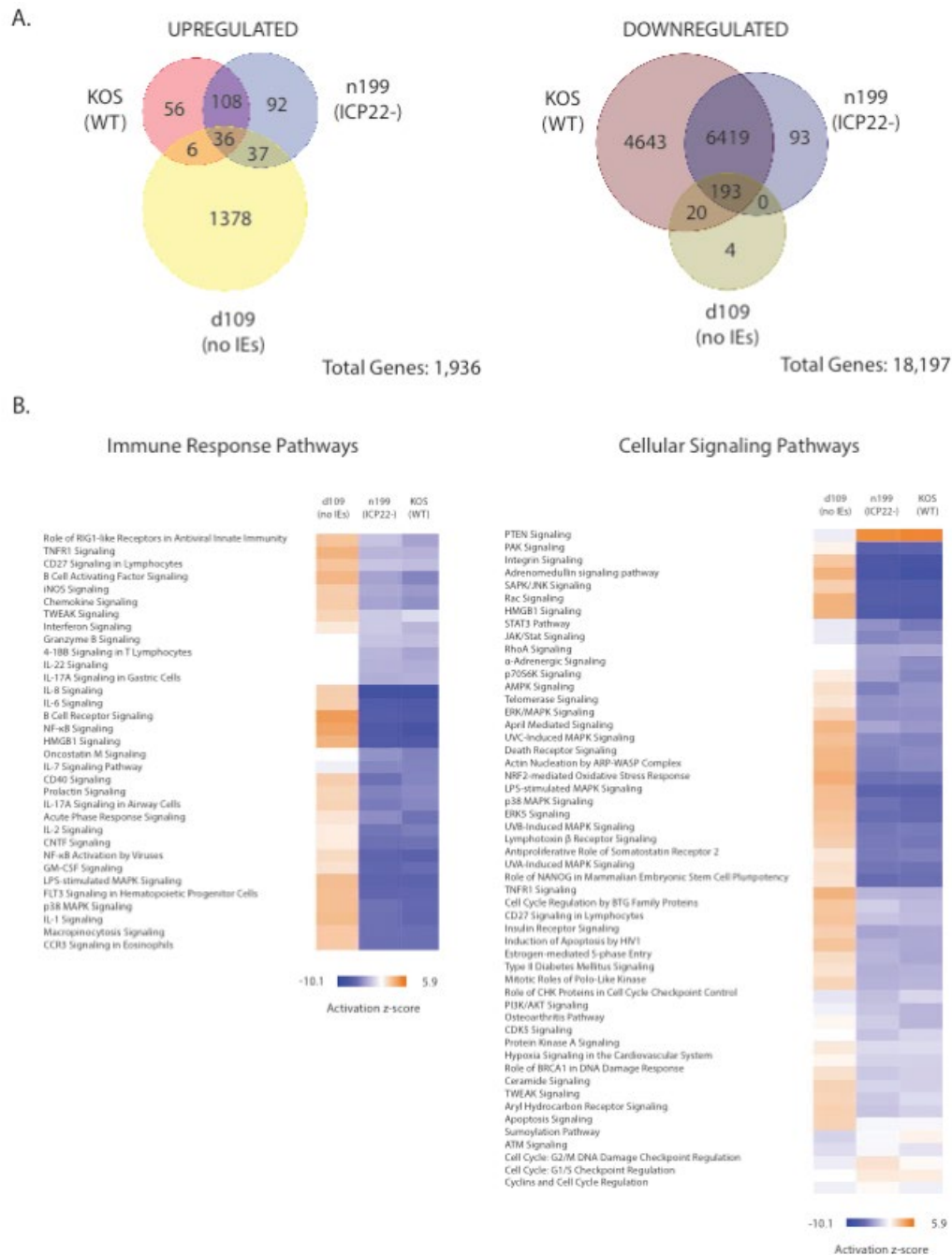


Figure 12. Differences in pathway regulation due to HSV-1 infection at 6 hours post infection. MRC5 cells were infected at an MOI of 5 with wild type (KOS) virus, ICP22 mutant virus (n199), IE mutant virus (d109), or TBS (Mock). RNA was harvested at 6 hpi. RNA-seq analysis, followed by differential expression analysis, was performed as described in the methods. EdgeR was used to calculate the logFC of reads mapped to genes in virus (KOS, n199, or d109) infected cells compared to mock infected. Data is based on 3 replicates. (A) Venn Diagrams of cellular genes significantly differentially expressed ($p < 0.05$) logFC of 1 or more (Upregulated) compared to mock and logFC of -1 fold or less (Downregulated) in cells infected with KOS, n199, or d109. (B) Ingenuity Pathway Core Analysis was performed on all 3 conditions: infection with KOS, n199, or d109. Cellular genes significantly differentially expressed ($p < 0.05$) were analyzed based on their logFC compared to Mock and p-value from DE analysis on 3 replicates in order to determine canonical pathways affected in each condition. Comparison analysis between the core analyses demonstrated differences in the degree of activation of different pathways

between the conditions and are represented by differences in z-score in the heat maps. Selected pathways involved in the immune response and cellular signaling that significantly differed among conditions are clustered and shown in the heat map.

The immune response pathways significantly altered by infection all follow a similar pattern at 6 hpi, being activated during infection with d109, and being repressed during infection with either n199 or KOS (Fig. 12B). These pathways include Interferon Signaling, Chemokine Signaling, Role of RIG1-like Receptors in Antiviral Innate Immunity, and B Cell Receptor Signaling (Fig. 12B).

Interestingly, by 6 hpi, PTEN signaling was activated in cells infected with either KOS or n199, in contrast to cells infected with d109 (Fig. 12B). PTEN is a phosphatase that plays an important role in the cell cycle. Activation of the PTEN signaling pathway by KOS and n199 may indicate an advantageous perturbation of the cell cycle that occurs during infection to benefit viral production.

Other cellular signaling pathways are activated in a similar pattern to immune pathways, indicating that IE proteins and/or their effects on the cell are required to shut down the host cell's innate response to HSV infection (Fig. 12B). Furthermore, many diverse pathways are repressed by KOS by 6hpi, suggesting that the downregulation of cellular transcripts is not specific to an antiviral response, but instead that repression of varied pathways indicates a general mechanism of downregulation of cellular transcription rather than a targeting of a specific cellular response.

Defining the Mechanism of Cellular Transcript Reduction

In order to address the mechanism by which ICP22 contributes to a downregulation of cellular transcripts, we investigated its effects on the distribution of RNA pol II on both cellular and viral genomes. RNA pol II has been shown to be depleted from cellular genes during infection with

HSV-1 (187, 188) and is known to be massively relocated to viral replication compartments (181, 195).

MRC-5 cells were infected with KOS, n199, or mock at an MOI of 10 and cross-linked at 6 hpi. In order to immunoprecipitate DNA cross-linked to RNA pol II, we used the antibodies 4H8 and 8WG16. Multiple antibodies were used due to the role of C-terminal domain (CTD) modifications of RNA pol II during transcription and during HSV-1 infection. RNA pol II with a hypophosphorylated CTD is typically recruited to gene promoters and serine-5 phosphorylated to initiate transcription. Next the serine-2 is phosphorylated, normally by cdk9, and elongation proceeds with RNA pol II with CTD phosphorylated serine-5 and serine-2 (259). During infection with wild-type HSV-1, the serine-2P/serine-5P RNA pol II decreases and serine-5P RNA pol II becomes more prominent. During infection with n199 the serine-2/serine-5 phosphorylated RNA pol II decreases, but the serine-5 phosphorylated form does not accumulate(185, 195, 231, 232). We wanted to determine whether this shift in phosphorylation state plays a role in the switch from cellular transcription to viral transcription upon infection. The 4H8 antibody will detect the CTD of RNA pol II when serine-5 is phosphorylated, whether alone or with ser-2 also phosphorylated. It can also detect hypophosphorylated RNA pol II. 8WG16 detects hypophosphorylated RNA pol II and also the CTD of RNA pol II when serine-5 is phosphorylated, but will not detect RNA pol II when the serine-2 of the CTD is phosphorylated as this will block the epitope of the antibody (236, 259). We used these antibodies in parallel in order to differentiate between the ser-5P/ser-2P and the ser-5P RNA pol II. We hypothesized that while viral genes can be efficiently transcribed by ser-5P RNA pol II, due in part to the actions of ICP22, cellular gene transcription is hindered by the loss of ser-

5P/ser-2P RNA pol II during HSV-1 infection due to a reliance on this form of RNA-pol II to transcribe cellular genes.

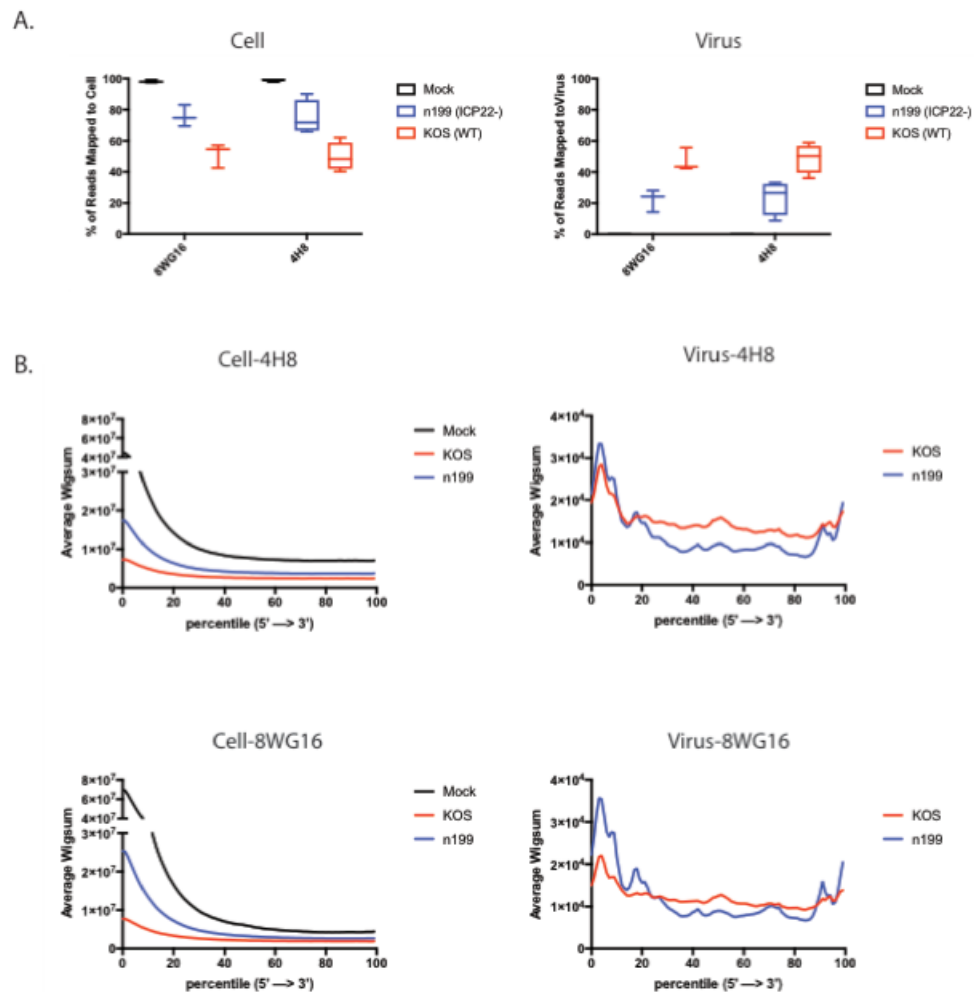


Figure 13. RNA polymerase II occupancy on cellular and viral genes during HSV-1 infection, 6 hpi. MRC5 cells were infected at an MOI of 10 with wild type (KOS) virus, ICP22 mutant virus (n199), or TBS (Mock). Infected cells were harvested at 6 hpi and processed for ChIP-seq as described in Materials and Methods. Two antibodies were used to immunoprecipitate RNA pol II: 4H8 and 8WG16. (A) Percent of total reads that map to either the cellular or viral genome in each condition. Data represents 3-4 biological replicates with standard deviation. (B) Gene Body Coverage Analysis on gene sets (Cellular and Viral). Represents the average distribution of RNA pol II across the entire gene. Wigsum represents coverage. Data is based on an average of 3 replicates.

As expected based on previous studies (187, 188), we observed an overall increase of RNA pol II on viral genes, complemented by a decrease on cellular genes (Fig. 13A). Consistent

with differences in overall gene expression (Fig. 10A), more RNA pol II was found to be associated with cellular genes, and less with viral genes, in cells infected with n199 compared to KOS infected cells. Overall the differences observed were similar between the two antibodies used, indicating that the relative distribution of RNA pol II between cellular and viral genes during infection is not altered by the phosphorylation state (Fig. 13A).

We have previously determined that at 6 hpi during infection with an ICP22 mutant (n199), RNA pol II accumulated on the start site of viral genes and decreased on gene bodies compared to during infection with WT (KOS), indicative of an elongation defect (258). In order to determine the average distribution of RNA pol II across cellular and viral genes from 5' to 3' we used the galaxy tool “gene body coverage” to calculate the average read coverage across the length of the genes in a set. Using a set of viral genes revealed a pattern on viral genes that was expected based on previous work. We observed less RNA pol II on gene bodies in cells infected with n199 as opposed to KOS, especially when viewed relative to accumulation on start sites, illustrating an elongation defect (Fig. 13B). This pattern is consistent between antibodies, although with 8WG16 we observed more accumulation at viral gene start sites in n199 infected cells compared to on viral genes than in KOS infected cells (Fig. 13B). The rise at the 3' end observed for the viral gene set is most likely due to the small distances between viral genes, making 3'ends close to the 5' of adjacent genes.

Compared to mock infected cells, cells infected with either KOS or n199 had less RNA pol II on both the start of genes and the gene body. The decrease at the start of cellular genes is more severe during infection with KOS compared to n199 (Fig. 13B). Interestingly, in both mock and n199 infected cells there is more RNA pol II detected toward start sites by 8WG16 compared to 4H8 and less on gene bodies by 8WG16 compared to 4H8. This indicates that ser-

2P/ser-5P RNA pol II may be more actively transcribing cellular genes during these conditions, coinciding with more cellular transcripts detected during these conditions (Figure 10).

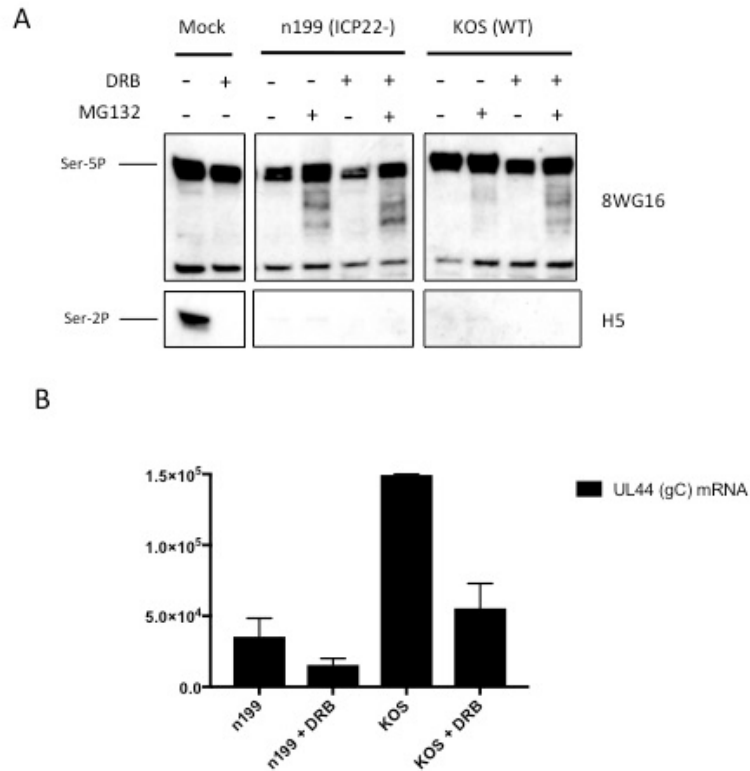


Figure 14. Kinase inhibitor DRB alters phosphorylation of RNA pol II. MRC5 cells were infected at an MOI of 10 with wild type (KOS) virus, ICP22 mutant virus (n199), or TBS (Mock). At 4 hpi DRB was added to indicated samples at a final concentration of 50 μ M. MG132 was also added to samples at 4 hpi when indicated. (A) Protein lysates were harvested at 6 hpi, run on a 4-15% SDS-PAGE gel and probed with the antibodies 8WG16 and H5. (B) RNA was harvested at 6 hpi and RT-PCR was performed to detect relative levels of UL44 transcripts. Data represents 2 biological replicates with standard deviation.

In order to further understand the role of RNA pol II CTD phosphorylation in HSV-1 transcription, we added the kinase inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) to infected cells at 4 hpi and harvested both protein and RNA at 6 hpi. We used two RNA Pol II antibodies to detect changes in RNA pol II phosphorylation. The antibody 8WG16 detects hypophosphorylated RNA pol II and RNA pol II with ser-5 phosphorylation. Hypophosphorylated RNA pol II will travel slightly farther than ser-5P RNA pol II when run on

a gel, differentiating the two forms. The H5 antibody detects ser-2P RNA pol II. In mock infected cells all three forms can be detected (Fig. 14A).

Upon addition of DRB in mock-infected cells, ser-2P RNA pol II was depleted and ser-5P RNA pol II decreased slightly. Infection with wild type (KOS) virus resulted in a depletion of ser-2P to at least the level of ser-2P in DRB treated mock-infected cells. Ser-5P did not decrease during KOS infection. Infection with ICP22 mutant (n199) virus also resulted in a depletion of ser-2P to at least the level of ser-2P in DRB treated mock-infected cells. Ser-5P RNA decreased slightly during n199 infection, compared to mock-infected cells. DRB treatment resulted in a similar reduction in Ser-5 phosphorylated RNA pol II in cells infected with n199 or KOS relative to the level in untreated infected cells (Fig. 14A).

To determine the effect that changes in RNA pol II CTD phosphorylation have on viral transcription, we compared viral transcript levels between the different conditions. We measured transcript levels of UL44, a viral late gene highly transcribed at 6 hpi. The level of transcription in different conditions correlated to the levels of ser-5 phosphorylated RNA pol II. Transcription levels decreased upon addition of DRB during infection with either KOS or n199. Additionally, transcription levels in cells infected with n199 and not treated with DRB were similar to levels in cells infected with KOS and treated with DRB (Fig. 14B). Addition of DRB to wild type infection therefore approximates transcription deficiencies observed in ICP22 mutants. This supports our hypothesis that ser-5P RNA pol II transcribes viral genes and that ICP22 is required for it to do so efficiently.

To further understand the dynamics of RNA pol II CTD phosphorylation during infection with KOS and n199, we added the proteasome inhibitor MG132 to some of the samples from 4hpi-6hpi. Addition of MG132 mostly rescued levels of ser-5 phosphorylated RNA pol II, but

did not rescue ser-2 phosphorylation in infected cells (Fig. 14A). Most likely this is due to ser-5P RNA pol II being present in cells prior to treatment, indicating that DRB treatment did not remove ser-5 phosphorylation but rather prevented any new phosphorylation. Moreover, the rescue of ser-5P RNA pol II by MG132 in cells infected with n199 and not treated with DRB could indicate that the loss of ser-5P RNA pol II during n199 infection is due to proteasomal degradation of stalled ser-5P RNA pol II on viral gene start sites that cannot proceed due to a lack of elongation factors normally recruited by n199.

Protein Interactions with ICP22 in Infected Cells

In addition to RNA pol II, other cellular proteins such as the FACT complex are relocalized during infection to viral genomes, and FACT coprecipitated with ICP22 indicative of protein-protein interactions (258). In order to determine the effect of ICP22 on the relocalization or actions of other cellular proteins, and possible other binding partners of ICP22, we immunoprecipitated FLAG-tagged ICP22 from cells infected with a virus expressing ICP22-FLAG at 6 hpi. In order to reduce the possibility that any interactions involved nucleic acid, we treated the samples with Benzonase, a nuclease that digests DNA and RNA, prior to immunoprecipitation. Proteins identified in two independent experiments with greater than 5 spectral counts and at least 4-fold enriched over the negative control were considered to be valid hits. Enrichment relative to the amount of ICP22 detected was calculated after normalizing for the molecular weight of each identified protein (Table 2). As we expected, the FACT complex was highly enriched compared to ICP22. Components of Casein Kinase 2 (CK2) were also detected in both. CK2 has been implicated in ICP22 function previously, with evidence supporting that it may be responsible for the nucleotidylation of ICP22 (179). Furthermore, CK2

has been found in complex with Spt16 and SSRP1. It has been hypothesized that Spt16 and SSRP1 can change the conformation of CK2 to target specific substrates for phosphorylation (261).

Many of the proteins highly enriched in the immunoprecipitation are involved in co-transcriptional processes. These proteins include an RNA helicase, DDX60, an ATPase chromatin remodeler, SMARCA1, the transcript release factor for RNA polymerase I, PTRF, and multiple components of the spliceosome including PRPF6, PRPF8, USP39, and SNRNP200. The association of ICP22 with these proteins supports our previous conclusions that at 6 hpi ICP22 promotes the efficient elongation of viral transcripts (258). We hypothesize that by recruiting these factors to viral genes, ICP22 can promote more efficient viral transcription and associated processes, contributing to a preservation of an open chromatin structure on viral genes and a lack of freely available proteins needed to sustain cellular transcription.

Although there is no direct evidence that ICP22 can bind to viral DNA, ICP22 can be detected in association with viral genomes isolated using click-chemistry (29, 181, 258). The one viral protein we found to interact with ICP22 was UL9, the HSV-1 origin binding protein. Although UL9 classically binds to the origins of replication and is not required for viral DNA replication after initial synthesis, UL9 continues to bind to viral DNA after the start of DNA replication (29). UL9 binding to viral DNA could play a role in viral transcription through association with ICP22, possibly by allowing ICP22 to indirectly associate with the viral genome.

Viral Proteins	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
ICP22	60/122	100%
UL9	27/65	24.6%
FACT Complex	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
Spt16	73/102	40.20%
SSRP1	42/39	29.60%
Casein Kinase 2	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
CSNK2A1	10/38	25%
CSNK2A2	15/28	27.5%
CSNK2B	9/12	23.4%
Spliceosome	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
PRPF6	15/37	12.2%
PRPF8	32/99	11.5%
USP39	10/19	11.7%
SNRNP200	23/81	10.1%
Transcription	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
PTRF	19/40	35.2%
SMARCA1	5/21	4.9%
RNA Helicase	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
DDX60	24/46	4.9%
Signal Sequence Binding	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
KPNA3	15/25	18.4%
KPNA4	11/28	16.7%
AP2M1	9/5	9%
DNA Replication	Spectral Counts (Rep 1/Rep2)	Enrichment Relative to ICP22 (normalized to MW)
FAM111A	15/32	17.20%

Table 2. Proteins interacting with ICP22 at 6 hpi. MRC5 cells were infected with wild type KOS or ICP22-FLAG at an MOI of 10. Cells were lysed and harvested at 6 hpi and lysates were treated with benzonase and bound to anti-FLAG beads. Proteins bound to the beads were eluted with 3x FLAG peptide and analyzed by mass spectrometry. Valid hits (included in table) were at least 4-fold enriched over the negative (KOS-infected) control.

3.5 DISCUSSION

During HSV-1 infection, cellular transcription decreases and RNA pol II relocates from cellular genes to viral genes (187, 188, 257). We have previously demonstrated a role for the viral

protein ICP22 in the elongation of viral transcripts through the recruitment of transcription elongation factors to viral DNA (258). In this study, we investigated the role of ICP22 in cellular transcription. We hypothesized that (a) during HSV-1 infection a reduction in cellular transcripts impairs the immune response to infection, (b) ICP22 contributes to the reduction of cellular transcripts, (c) ICP22 recruits elongation factors to viral genomes to subvert blocks to cellular transcription mechanisms, and (d) ICP22 therefore increases efficiency of viral transcription at the direct expense of cellular transcription. Our data support the hypothesis that the actions of ICP22 not only increase viral transcription, but also contribute to the repression of cellular transcription. Furthermore, we present a strategy by which HSV-1 may directly disrupt cellular transcription processes while providing an alternative means of transcription for viral genes.

ICP22 and Cellular Transcripts

Infection with wild type KOS or ICP22 mutant n199 results in the downregulation of a wide variety of cellular genes, most likely due to many viral factors and processes involved in the downregulation of cellular transcription. This includes viral proteins such as VHS and ICP27, which reduce levels of cellular mRNA, and also processes such as viral DNA replication, which can be coupled to viral transcription and are required for the virus to transcribe late genes and flood the cell with viral transcripts. We determined that ICP22 also has a role in downregulating cellular transcription in infected cells. Overall cellular transcription was more reduced in cells infected with wild type KOS virus than in cells infected with ICP22 mutant n199 virus. Pathways affected included those related to the immune response, supporting the observation that the ICP22 mutant virus n199 is less virulent during in vivo infection in mice (184).

RNA pol II Modifications

In contrast to prior studies, we found no evidence to support the interaction of ICP22 and cdk9 (198, 199, 249). There is, however, evidence that cdk9 is associated with viral genomes during infection. In fact, recent work suggests that ICP4 may be required for the recruitment of cdk9 to viral genomes (29). There has been debate as to whether ser-2P by cdk9 is required for efficient viral transcription. Several studies suggest that the inhibition of cdk9 results in a decrease in viral transcription (196, 197). These studies have not all addressed the possibility that cdk9 may also phosphorylate ser-5P in the context of viral infection, or that inhibitors that target cdk9 may have off-target effects on kinases that phosphorylate ser-5P in infected cells. We determined that using the inhibitor DRB can reduce the levels of ser-2P, but can also reduce levels of ser-5P, especially in HSV-1 infected cells. We observed no effect on the already undetectable levels of ser-2P in infected cells treated with DRB. Instead, transcription levels correlated with levels of ser-5P in infected cells, due to treatment with DRB or infection with the ICP22 mutant n199. We therefore propose that viral transcription mainly relies on ser-5P RNA pol II and that doing so allows for the efficient transcription of viral genes at the expense of cellular genes. ICP22 is essential to this strategy as it can recruit transcription elongation factors and RNA processing factors to viral genomes. Interestingly, levels of ser-5P RNA pol II are reduced in the ICP22 mutant virus. This reduction could be due to a direct action of ICP22 or possibly be the result of a lack of efficient elongation leading to stalled ser-5P RNA pol II being degraded (170, 232).

Recruitment to Viral Genomes

ICP22 interacts with many cellular proteins at 6 hpi, many of which have a role in transcription elongation and RNA processing. Some of these, such as CK2 components, the FACT complex,

and SMARCA1, have been found to also associate with viral DNA at 6 hpi (29). CK2 is a kinase that has also been determined to interact with the FACT complex and the viral protein ICP27 (163). CK2 may modify ICP22 and/or ICP27, or be hijacked by the virus to modify other proteins. This would be interesting to explore further.

ICP22 interacted with the splicing factors PRP6, PRP8, USP39, and SNRNP200. Most HSV-1 genes are not spliced, but splicing factors may have other roles in viral transcription. The pre-mRNA processing factors PRP6 and PRP8, found in this study to interact directly with ICP22, are associated with viral DNA throughout infection (181). PRP6, PRP8, USP39, and SNRNP200 play roles in the assembly of the U4/U6.U5 tri-SNP (262). During HSV-1 infection, the assembly of the U4/U6.U5 tri-SNP is inhibited by ICP27 in order to inhibit the splicing of cellular mRNA (164). ICP22 may recruit these proteins so that HSV-1 can repurpose these now available RNA-processing proteins to help process viral mRNAs and that ICP22 may recruit them. Therefore, HSV-1 preventing assembly of the spliceosome may have a dual role in reducing the levels of cellular transcripts while promoting the efficient transcription of viral transcripts.

We have not found evidence to support ICP22 as a DNA binding protein, leaving open the question of how ICP22 itself gets recruited to viral genomes. In this study we found that at 6 hpi ICP22 interacts with UL9, the HSV-1 origin binding protein. Although UL9 is only required for the initiation of viral DNA replication, levels of UL9 do not decrease after the onset of HSV-1 replication and can be found associating with viral genomes at 6 hpi (84, 263–265). Functions for UL9 other than initiating viral DNA synthesis have yet to be determined. We hypothesize that UL9 may remain bound to viral DNA in order to recruit ICP22 and possibly other proteins directly to viral DNA to enhance viral DNA transcription after viral DNA replication.

3.6 MATERIALS AND METHODS

Cells and Viruses

Experiments were performed using MRC5 (human fetal lung) or Vero (African green monkey kidney) cells obtained from and propagated as recommended by ATCC. The viruses used in this study include HSV-1 wild type KOS and mutants made with the KOS background. The mutant viruses used include d109, n12, n199, and 5dl1.2. A virus in which a FLAG tag has been inserted after the start codon of US1, ICP22-FLAG was also used.

Co-immunoprecipitation and Mass Spectrometry

MRC5 cells were grown to confluence in a 150 mm plate. Cells were infected with ICP22-FLAG or KOS (control) at an MOI of 10 in tris-buffered saline (TBS) for 1 hour before removing inoculum and rinsing with TBS. Cells were then incubated at 37°C in DMEM containing 2% FBS for 6h. After incubation, cells were rinsed with ice-cold TBS plus 0.1 mM Tosyl-L-lysyl-chloromethane hydrochloride (TLCK). Cells were then lysed at 4°C for 30 min in 1.5 mL lysis buffer (50 mM Tris-HCL [pH 7.4], 420mM NaCl, 2mM MgCl₂ 1% Triton x 100, 1mM EDTA, 1X Roche protease inhibitor cocktail, 1X Roche PhosSTOP). Lysates were then centrifuged at $1.8 \times 10^3 g$ for 10 min at 4°C and supernatants filtered through a 100µm cell strainer (Corning). Samples were incubated in 10 µL Benzonase (Novagen) at this step for 10 minutes. Samples were then combined 1.1:2 with Binding Buffer (50 mM Tris-HCL [pH 7.4], 1% Triton x 100, 1mM EDTA, 1X Roche protease inhibitor cocktail, 1X Roche PhosSTOP) and added to ANTI-FLAG M2 Magnetic Beads (Sigma). Immunoprecipitation was carried out according to the manufacturer's protocol with a few modifications. Briefly, samples were bound to the Anti-Flag beads overnight and following a series of washes with Tris-buffered saline (50 mM Tris HCl [pH

7.4], 150 mM NaCl, 1X Roche protease inhibitor cocktail, 1X Roche PhosSTOP). Bound proteins were eluted using the recommended concentration of 3X FLAG Peptide (Sigma). Eluted protein was sent to MSbioworks, LLC, Ann Arbor, Michigan for Mass Spectrometry. Proteins 4-fold enriched over KOS control and present in both replicates were considered significant.

Inhibitor Assay

MRC5 cells were seeded into 60mm dishes at a density of 2×10^6 cells per dish, infected with n199 or KOS at a MOI of 10 PFU/cell, and incubated at 37°. Cells were then incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for 4 hours. DRB was added to a final concentration of 50 μ M to cells at 4hpi. Protein and RNA was harvested 6 hpi. To collect protein samples were rinsed with ice-cold TBS plus 0.1 mM tosyl-l-lysyl-chloromethane hydrochloride (TLCK) before being scraped into 2 \times SDS-PAGE sample buffer. Samples were boiled for 10 min before analysis. RNA was collected using the Ambion RNeasy-4 PCR kit using the included protocol.

Protein samples were run on a 4-15% precast gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (Amersham), and probed with the following primary antibodies: mouse anti-RNA pol II 8WG16 (abcam, ab817, 1:500) and mouse anti-RNA pol II H5 (BioLegend; 1:500). After incubation with anti-mouse-horseradish peroxidase (Promega), bands were visualized using ECL Prime Western blot detection reagent (Amersham).

Isolated RNA was quantified using an Agilent eukaryotic RNA Nano kit and a 2100 Bioanalyzer. 500ng RNA was reverse transcribed using an Epicentre Kit (RT80125K). Quantitative real-time PCR was carried out as described previously (62). Transcript quantity was determined based on the amplification of HSV-1 gene UL44 using the gCds primers (mike

paper). Two biological replicates were performed.

RNA sequencing (RNAseq)

MRC5 cells were seeded into 60mm dishes at a density of 2×10^6 cells per dish, infected with n199 or KOS at a MOI of 5 PFU/cell, and incubated at 37°. At the appropriate time post infection, RNA was isolated using the Ambion RNaqueous-4PCR kit using the included protocol. Total RNA was quantified using the Agilent RNA 6000 Nano Kit. RNA-Sequencing libraries were generated from 1-2 µg RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #E7490 and #E7420). Libraries were quantified using the Agilent DNA 7500 Kit, and samples were mixed together at equimolar concentration. Illumina HiSeq 2500 was carried out at the Tufts University Core Facility.

RNA-seq was mapped to the human genome (hg38) using HTseq, after which unmapped reads were extracted and mapped to the HSV-1 genome (KT899744). Differential Expression Analysis was performed using EdgeR, normalizing for sequencing depth instead of the default TMM normalization. IPA core analysis was performed on the log fold change and p-values of significant genes for each sample. Comparison analysis was then performed across time points. Pathways included in the figures were divided into Immune Response Pathways (Cellular Immune Response, Cytokine Signaling, Humoral Immune Response, Pathogen-Influenced Signaling) and Cellular Signaling Pathways (Apoptosis, Cell Cycle Regulation, Cellular Stress and Injury, Intracellular and Second Messenger Signaling, Transcriptional Regulation).

Chromatin Immunoprecipitation Sequencing (ChIPseq)

MRC5 cells were infected with virus at a MOI of 10 PFU/cell, and incubated at 37° for 2.5 or 6 hours, as indicated. ChIP-seq was performed as described previously, with the following modifications. Cell extracts used for immunoprecipitation were prepared from 1.75×10^7 cells in 150mm tissue culture dishes, or 7×10^7 cells in 600 cm² square tissue culture dishes and divided into 4 samples after sonication. The number of washes with LiCl wash buffer was increased to 7. Immunoprecipitations were performed using either the antibody 4H8 (abcam, ab5408) or 8WG16 (abcam, ab817). For each sample 10-20 ng DNA was used to create sequencing libraries using the NEBNext Ultra II DNA Library preparation kit (NEB #E7103S). Libraries were quantified using the Agilent DNA 7500 Kit, and samples were mixed together at equimolar concentration. Illumina HiSeq 2500 was carried out at the Tufts University Core Facility.

ChIP-seq was mapped to the human genome (hg38) using HTSeq, after which unmapped reads were extracted and mapped to the HSV-1 genome (KT899744). Data were analyzed using GALAXY. Bam files were converted to bigwigs, normalizing counts based on total reads in the sample and for viral alignments number of viral genomes in addition to total reads in the sample. Gene Body Coverage was performed on the bigwigs to generate the average coverage for each percentile of the length of each gene. In order to calculate gene body coverage for viral genes, a set of selected viral genes that were determined to not overlap the 5' or 3' end of other viral genes (RL2, UL19, UL21, UL25, UL29, UL37, UL38, UL42, UL44, UL47, UL48, UL49, UL54, RS1, US4, US6, UL4, UL18, UL30, UL35, UL36, UL39, UL46, US1, US7). The cellular gene set GRCh38.79 was used to calculate gene body coverage for cellular genes. For each 8WG16 sample the gene body coverage is an average of 3 replicates. For each 4H8 sample the gene body coverage is an average of 4 replicates.

4.0 SUMMARY AND DISCUSSION

4.1 SUMMARY OF THESIS

Transcription in cells infected with HSV-1 is greatly regulated by viral proteins. As viral transcripts accumulate in infected cells, cellular transcripts decrease, indicating that HSV-1 exerts regulatory control over cellular transcription as well as viral transcription. The transcription cascade that characterizes the expression of viral genes is dependent on the two recognized viral transcription activators VP16 and ICP4. There is also evidence that the IE proteins ICP27 and ICP22 are involved in the regulation of viral transcription. Previously, ICP22 was thought to affect late gene transcription, but the mechanism behind this activity was not defined. We undertook an investigation of the role of ICP22 in regulating viral and cellular transcription during HSV-1 infection.

In Chapter 2 we determined that the FACT complex is recruited to viral DNA by ICP22. We affinity purified viral genomes at 6 hours post infection with either wild type virus or an ICP22 mutant virus and determined that the FACT complex members do not associate with viral genomes in the absence of ICP22. Moreover we engineered a virus that expresses a FLAG-tagged ICP22 in order to affinity purify ICP22 and determine that ICP22 interacts with both members of the FACT complex at 3 and 6 hours post infection. We hypothesized that ICP22 and the FACT complex might play a role in viral transcription elongation. We tested our hypothesis

with RNA-seq and ChIP-seq experiments and determined that in the absence of ICP22, transcription of viral genes decreases at 6 hours post infection and that RNA pol II does not proceed down viral gene bodies. We therefore concluded that ICP22 is responsible for recruiting the FACT complex (and other proteins) to viral genomes in order to promote the efficient elongation of viral transcripts.

In Chapter 3 we proposed a model by which HSV-1 represses cellular gene expression and investigated how ICP22 contributes to this strategy. We compared changes to cellular transcription induced by infection with either wild type virus or ICP22 mutant virus and concluded that infection with the ICP22 mutant virus resulted in both the repression of fewer cellular genes and repressed cellular genes to a lesser extent. Furthermore, ChIP-seq experiments revealed that RNA pol II occupancy is greater on cellular genes infected with the ICP22 mutant compared to wild type. By repeating the affinity purification of ICP22-FLAG presented in Chapter 2 and analyzing proteins purified by mass spectrometry, we confirmed that ICP22 interacts with the FACT complex. We also discovered new interactions. We determined that ICP22 interacts with the viral protein UL9 and may be recruited to viral DNA through this interaction. Finally, we observed a correlation between the rate of viral gene transcription and the amount of Ser-5P present in infected cells. We therefore propose that ICP22 contributes to the repression of cellular transcription during HSV-1 infection by recruiting cellular factors to viral genomes. Specifically, ICP22 aids in robust viral transcription with Ser-5P RNA pol II by recruiting cellular transcription elongation factors to viral genomes, allowing the virus to alter RNA pol II modifications in a way that is unfavorable to cellular transcription.

Taken together, our studies present a role for ICP22 in transcription regulation in cells infected with HSV-1. Further implications of this research are discussed below.

4.2 GENERAL DISCUSSION

Why is viral DNA preferentially transcribed?

Viral and Cellular genes are both transcribed by RNA pol II, yet during HSV-1 infection RNA pol II is mostly associated with viral genes and viral transcription exceeds cellular transcription (Chapter 3). This raises the question of how HSV-1 represses cellular gene expression while still maintaining robust viral transcription. We propose a model in which ICP22 plays a key role in sustaining viral transcription at the expense of cellular transcription:

1. Viral DNA enters the nucleus and is detected by the cell, leading to an initial repression of the viral genome and recognition by IFI16.
2. VP16 and ICP0 remove chromatin and other repressive cellular factors from viral DNA to allow for recognition by ICP4.
3. ICP4 binds to exposed viral DNA and multimerizes, ensuring ICP4 binds strongly to viral, rather than cellular, DNA.
4. ICP4 recruits initiation factors to viral DNA, promoting robust viral transcription initiation and sequestering these factors away from cellular genes.
5. Viral DNA replication begins and also promotes viral transcription by providing more templates and keeping viral DNA accessible to transcription factors through the removal of histones from replicating DNA. This is supported by the observed coupling of viral replication and transcription (85). We propose that UL9 binds to ssDNA near replication

forks and recruits ICP22 and associated elongation factors to these sites of active viral DNA replication and transcription.

6. Infection also leads to a loss of Ser-2P RNA pol II and an accumulation of Ser-5P RNA pol II. The loss of Ser-2P RNA pol II is independent of ICP22, and may be due to the direct or indirect actions of HSV-1, leading to a lack of proper elongation and termination of cellular transcripts. ICP22 is able to recruit transcription elongation and RNA processing factors to Ser-5P RNA pol II, and recruits them to viral DNA rather than cellular DNA due to its binding to viral DNA via UL9. Furthermore, Ser-5P RNA pol II is already massively relocated to viral DNA due to the actions of ICP4 and active viral DNA replication. ICP22 therefore promotes efficient elongation of viral transcripts, ensuring robust viral gene transcription.
7. The cycle of robust viral transcription and repressed cellular transcription is self-perpetuating because without proper transcription the cell is unable to mount an immune response to the virus that could repress further viral transcription.
8. Other factors also contribute to the reduction of the amount of cellular mRNA that gets translated:
 - a. VHS-mediated degradation of mRNA
 - b. ICP27 inhibition of splicing

Impact of RNA pol II Modification

Our studies support the conclusion that RNA pol II with ser-2P is degraded, or that ser-2P is inhibited, during infection with HSV-1 (195). Moreover, we suggest that ser-5P RNA pol II is all that is required to transcribe viral genes as ICP22 can recruit the factors that would normally

only dock on ser-2P RNA pol II. This conclusion is supported by recent data suggesting that ICP4 is required for the recruitment of cdk9 to viral genomes, but not for the recruitment of factors that normally associate with ser-2P such as elongation and RNA processing factors (29, 266).

Why, then, is cdk9 present if ser-2P is unnecessary or even detrimental to viral infection? One hypothesis is that cdk9 phosphorylates ser-5 rather than ser-2 in infected cells. This hypothesis would explain why it is recruited to viral genomes by ICP4, which is responsible for the promotion of transcription initiation and also recruits TFIIH to viral genomes. Cdk9 has been found to be required for the Tat-Activated elongation of Human Immunodeficiency Virus (HIV) viral transcripts (267). Interestingly, during HIV infection cdk9 may associate with the initiation complex and, along with TFIIH subunit cdk7, phosphorylate ser-5 rather than ser-2 (268).

We propose that the lack of ser-2P during infection is beneficial to productive viral infection by preventing elongation and termination factors from being available for cellular transcription. Recent work has suggested that the termination of cellular mRNA is aberrant in infected cells, which would support a lack of ser-2P RNA pol II (260). While we suggest that ICP22 can recruit elongation factors and RNA processing factors to viral DNA in the absence of ser-2 phosphorylation, we do not have evidence that ICP22 recruits termination factors. ICP27 may possibly be required for recruiting these factors in the absence of ser-2P RNA pol II and this could be further investigated.

A Role for UL9 Late in Infection

UL9 is the HSV-1 origin binding protein and has been studied for its role in viral DNA replication. We hypothesize that UL9 plays a role in allowing ICP22 to bind to viral DNA.

Although UL9 is not required for continued viral DNA synthesis past the onset of replication, UL9 remains associated with viral DNA (29, 84, 85, 265). Furthermore, we found that ICP22 interacts with UL9 at 6 hours post infection (Chapter 3). In order to determine what role UL9 plays at later times during infection, a virus could be engineered that expresses an affinity-tagged UL9. We could then use ChIP-seq to determine how UL9 is associated with viral DNA during infection, if it binds to promoters, gene bodies, or to specific genes. We could also immunoprecipitate the tagged UL9 and identify the proteins that interact with it during infection.

Temperature-sensitive mutants could be employed to determine which factors rely on UL9 to bind to viral DNA. UL9 binds ICP8 to promote viral DNA synthesis and may play a similar role in promoting viral gene transcription by binding to ICP22 and other factors (269, 270). Since UL9 may bind to ssDNA, it is plausible that UL9 is highly attracted to viral DNA that is being replicated and transcribed, and can therefore recruit transcription regulators like ICP22 to where they are most needed (271, 272). Using a temperature sensitive mutant at permissive vs nonpermissive temperatures and affinity-purifying viral DNA, as was done in Chapter 2, could reveal which proteins require UL9 in order to associate with DNA. Coupled with ChIP-seq and co-immunoprecipitation experiments, this study could help determine the role of UL9 later in infection.

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